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IONIC SPECIES IN ORTHOPHOSPHATE ABSORPTION BY BARLEY ROOTS[1]

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Absorption of phosphate by higher plants has been considered to involve the $H_2PO_4^-$ ion (7, 10). This conclusion has been based on simple correlations of phosphate sorption with $H_2PO_4^-$ concentration.

Results obtained from this study indicate that both $H_2PO_4^-$ and $HPO_4^=$ are absorbed under aerobic conditions by excised barley roots, and that the absorption of both ion species is competitively inhibited by hydroxyl ion. Expressions are derived that are in quantitative agreement with the observed absorption of phosphate. Radioactive phosphate was used as a tracer as well as a very low root mass per unit volume, and a sorption period of short duration.

MATERIALS AND METHODS

Excised roots of *Hordeum vulgare*, variety Atlas 46, were grown essentially as described by Epstein and Hagen (3). Twenty grams of seed were soaked for 24 hours in one liter of continuously aerated demineralized water at 24° C in a dark chamber. They were then rinsed in demineralized water and distributed on boiled cheesecloth supported on a stainless steel screen having a diameter of 12 cm. Each screen was made with four flaps which were turned down over the top of a four-liter beaker containing 1×10^{-4} M $CaSO_4$ solution, so that the seeds rested one centimeter above the solution. Another steel screen was placed over the seeds and this in turn was covered with boiled cheesecloth. Sintered glass aerators were placed in the solution and the beaker assembly was then covered with a watch glass and placed in a dark chamber. Seedling roots were grown five days in the continuously aerated $CaSO_4$ solution maintained at 24° C in darkness. Forty-four hours after planting, the glass and cheesecloth covers were removed; and three days after planting, the seedlings were removed from the solution, rinsed twice with demineralized water and placed in renewed $CaSO_4$ solution. During each of the latter two operations, the seedlings were exposed to low intensity incandescent light for about three minutes. Healthy roots 10 to 15 cm long were obtained by this procedure. These were excised with clean shears about 5 mm below the seeds. Excised roots were rinsed and placed in 3 liters of demineralized water.

In preliminary experiments, one liter of solution was found necessary for each one-gram portion of roots to maintain the concentration of phosphate essentially constant over an absorption period of three hours. Thus, one-liter solutions having the appropriate total phosphate concentration and specific activity were made up in one-liter Erlenmeyer flasks. The pH of the solutions was adjusted to the desired value with 0.1 *N* KOH or 0.1 *N* HCl. Flasks were placed in a water bath at 30° C and the solutions, under constant aeration, came to the temperature of the water bath.

A calculated amount of non-radioactive phosphate was added to the P^{32} in 0.04 *N* HCl, obtained from the Oak Ridge National Laboratory, with a specific activity of 40,000 mc/gm P. The solution was then evaporated to dryness under an infrared lamp to remove the acid and the residue was dissolved in demineralized water to prepare the phosphate stock solution. Phosphate absorption in three hours was not affected by levels of specific activity up to 3200 mc P^{32}/gm P. In all other experiments the specific activity was between 0.32 and 3.2 mc/gm P.

Excised roots were removed from the demineralized water in which they had been suspended for a period of 30 minutes and were blotted on cheesecloth to remove adhering water. One-gram portions were weighed out and placed in 50 ml demineralized water at 24° C in a test tube. When the solutions contained in flasks previously placed in a water bath reached a temperature of 30° C, the water in each test tube containing one gram of excised roots was decanted and 50 ml demineralized water added. At zero time roots were transferred, en masse, to phosphate solutions in one liter flasks. From 60 to 120 minutes elapsed from excision of roots to placement in the absorption flasks.

The flasks were returned to the water bath and aerated continuously during the absorption period with CO_2-free air at a rate of approximately 12 l/hr × l solution. In preliminary work, bicarbonate ion at a concentration of 4×10^{-4} M did not interfere with absorption of phosphate from solutions at 5×10^{-6} M phosphate, and pH levels of 5.0 and 7.7. However, removal of CO_2 was adopted as a standard procedure in view of the possibility that bicarbonate might conceivably interfere with absorption of other ions.

Flasks were removed from the bath within three minutes after addition of roots and the solutions readjusted to the desired pH value. Solutions having high total phosphate concentration required infre-

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quent adjustment during absorption periods, once each half-hour being sufficient. At low total phosphate concentrations and all pH values, however, adjustments had to be made at intervals of 15 minutes. In solutions having total phosphate concentrations between 10^{-6} to 10^{-5} M, and with initial pH values ranging from 4.0 to 5.0, the pH tended to increase up to one-half unit over the first 30 minutes of the three-hour absorption period. Under similar conditions of phosphate concentrations, and in solutions having initial pH values ranging from 6.0 to 8.0, the pH tended to decrease as much as one-half unit.

At the end of the absorption periods, 50-ml portions of the solutions were saved for assay of activity and the roots were rinsed with demineralized water to remove adhering solution. The lots of roots were dried under an infrared lamp preparatory to measurement of radioactivity. Activity in samples of the stock solutions, with the same geometry as the root samples, were measured with a Geiger-Mueller tube. No correction for self-absorption was necessary because of the high energy beta emission of P^{32} and the range of counting was so high that the effect of the presence of K^{40} was negligible. The activity remaining in the solutions was measured. The extent of recovery of activity ranged from 95 to 100 % of that initially present.

Each point expressing the absorption under conditions of known phosphate and hydrogen ion concentrations, figures 1 to 3, is a mean value obtained from five experiments.

Basic Equations and Results

Experimental design was based on the assumption that absorption of ions involves formation of reversibly dissociable intermediates. Further, the rate of association and dissociation of the active intermediates is very rapid when compared with the irreversible reaction rate of the breakdown of the intermediate. These assumptions are formally expressed in the following equations:

$$(1) \qquad R + M_{outside} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR$$

$$(2) \qquad MR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R' + M_{inside}$$

where R represents a metabolically produced site, M the ion, MR the active intermediate, and k the rate constant for each reaction. The k_3 is the rate-limiting step of absorption. Such assumptions have been shown to be valid for the absorption of alkali cations (3), as well as the halides (2).

Elementary application of these assumptions to experimental results requires a steady-state approximation. Within the limits of the experimental conditions a steady-state was shown to exist by a linear rate of phosphate absorption throughout an absorption period of three hours. Further, the absorption of phosphate by barley roots has been shown to be essentially irreversible (8), the k_4 is negligible.

A velocity equation may be derived from equations *1* and *2* which is entirely analogous to the steady-state analysis described by Michaelis and Menten (11) for kinetic studies of enzyme reactions. Lineweaver and Burk (9) pointed out the velocity equation is linear in form upon taking the reciprocal of both sides, as shown by equation *3*;

$$(3) \qquad 1/v = \frac{K_M}{V_{max}(S)} + 1/V_{max}$$

In the analogous expression describing absorption, substrate concentration, (S), will be expressed as $[\Sigma P]$ for total phosphate concentrations or in certain cases as the concentration of a specific ion species of orthophosphate. The observed absorption of phosphate is denoted by v, and the maximum absorption at infinite substrate concentration as V_{max}. The K_M is equal to the apparent dissociation constant of an activated intermediate of phosphate, RM.

If the process of phosphate absorption at steady-state involves a single first order reaction, described by the velocity equation, a double reciprocal plot of the variables, v and $[\Sigma P]$, would result in a straight line. That the experimental measurements cannot be described by a single first order reaction is evident from figure 1. To test whether more than one independent reaction is acting on a single substrate, or on different substrates which are in a non rate-limiting equilibrium, a plot of absorption, v, against $v/[\Sigma P]$ was made to permit separation of the component reactions by graphical methods (6), and figure 2. The observed absorption at pH 4.0 and 7.0, curve c, is composed apparently of two first order reactions, curves a and b, with both reactions acting simultaneously and independently on orthophosphate, per se. The two reactions may be resolved from the observed curve by the use of polar coordinates. In the case where the dissociations constant, K_M, of one reaction differs widely from the other, an extrapolation to the intercept of both coordinates may be made for the first order reaction that is approximated at low values of $v/[\Sigma P]$ as well as that reaction approximated at relatively high values of $v/[\Sigma P]$. The highest extrapolated

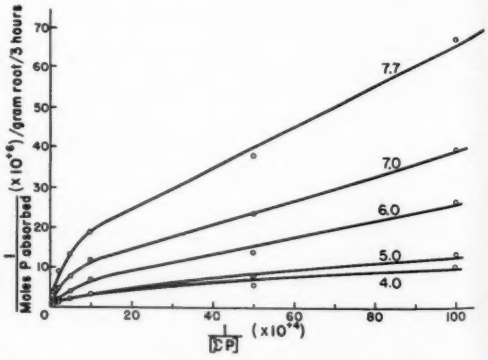


Fig. 1. Double reciprocal plot of phosphate absorption at varying pH.

TABLE I

ABSORPTION OF PHOSPHATE AT VARYING pH VALUES AS % OF ABSORPTION AT pH 4.0

[ΣP]	pH			
	5.0	6.0	7.0	7.7
	%	%	%	%
1×10^{-6}	89.2	37.8	25.8	15.0
2×10^{-6}	85.7	39.4	23.4	14.4
5×10^{-6}	117.4	48.9	29.7	19.4
1×10^{-5}	125.0	61.3	27.5	16.8
2×10^{-5}	134.0	81.4	34.5	16.9
1×10^{-4}	114.7	115.3	64.6	18.9

value on both the ordinate and the abscissa is a summation of the values contributed by both reactions. Thus, a subtraction of the extrapolated values on the ordinate, as well as the values on the abscissa, establishes the points which are determinate to the two curves. The latter procedure was considered to be applicable in the present instance.

The maximum absorption, V_{max}, is given directly by the intercept with the ordinate, figure 2. At pH 4.0 the individual reactions a and b comprising the observed absorption differ in their V_{max} by one factor of ten. Further, at pH 7.0 both first order reactions are inhibited, but at infinite phosphate concentrations the maximum absorption, in each case, is the same as that at pH 4.0. Since $V_{max} = Rk_3t$, it is evident that neither hydrogen ions nor hydroxyl ions are involved in irreversible changes in the binding sites, R_a^+ or R_b^+, or the rate constants. Thus, a strictly competitive interference of phosphate absorption is shown implicitly by a factor associated with increasing pH values.

Two different sites are involved evidently in phosphate absorption. The ion species of orthophosphate actively involved, and the factor associated with increasing pH which exhibits a competitive inhibition of phosphate absorption, may be established by qualitative inspection of the results.

Qualitative inspection of figure 2 shows the absorption curves can be best represented as two processes, as previously stated. Further, a number of mechanisms have been eliminated as a description of the system by not being consistent with the experimental results. A system of two species of orthophosphate absorbed through separate types of sites, with differing pH-activity relationships, however, is qualitatively consistent with the results in the following manner. At higher [ΣP] values, the site with the greatest V_{max}, curve a, contributes the largest percent of the total phosphate absorbed, figure 2. At the high value of [ΣP] of 1×10^{-4} M, absorption approximately parallels the percent of the singly charged species, $H_2PO_4^-$, with varying pH, tables I and II. Thus, $H_2PO_4^-$ is a logical substrate for the process of absorption denoted by curve a, figure 2. At the low concentrations of total phosphate the decrease in absorption is greater than the decrease in percent $H_2PO_4^-$ at the intermediate pH values. Since this decrease in absorption occurs despite an increase of $[HPO_4^=]$ with increasing pH values, table II, a pH effect, per se, must be invoked. However, the rate of decrease of absorption becomes less than the rate of decrease of $[H_2PO_4^-]$ with unit increases of pH values from 5.0 to 7.7, table I. With these increasing pH values, the concentration of $HPO_4^=$ increases to a significant value, and a large percent of the total concentration of phosphate. Absorption of $HPO_4^=$ through its respective site is consistent with these results. The process for which $HPO_4^=$ is the substrate is denoted by curve b, figure 2. The strictly competitive nature of the pH-activity effect on the absorption of an anion, phosphate, figure 2, suggests that hydroxyl ion, OH^-, is the competitive ion.

The above considerations result in a system described by an effect of pH on the species of phosphate ion in solution:

$$(4) \quad H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \rightleftharpoons H^+ + HPO_4^= \rightleftharpoons H^+ + PO_4^{\equiv}$$

the combination of two active ion species with their respective binding sites;

$$(5) \quad R_a^+ + H_2PO_4^- \rightleftharpoons R_aH_2PO_4$$

$$(6) \quad R_b^+ + HPO_4^= \rightleftharpoons R_bHPO_4^-$$

and an additional effect of pH expressed by a competitive interference by the hydroxyl ion, OH^-, for both binding sites;

$$(7) \quad R_a^+ + OH^- \rightleftharpoons R_aOH$$

$$(8) \quad R_b^+ + OH^- \rightleftharpoons R_bOH$$

EXPRESSION OF EXPERIMENTAL OBSERVATIONS

The effective concentration of the species of orthophosphate ions in solution is limited by the ionization equilibria, as shown by equations *9*, *10*, and *11*.

$$(9) \quad K_{1h} = \frac{[H_2PO_4^-][H^+]}{[H_3PO_4]}$$

$$(10) \quad K_{2h} = \frac{[HPO_4^=][H^+]}{[H_2PO_4^-]}$$

$$(11) \quad K_{3h} = \frac{[PO_4^{\equiv}][H^+]}{[HPO_4^=]}$$

Different binding sites, or carriers, are involved in the absorption of $H_2PO_4^-$ and $HPO_4^=$. Further OH^-

TABLE II

SPECIES OF PHOSPHATE IONS AT VARYING pH VALUES AS % OF ΣP. THE RATIO OF SPECIES IS INDEPENDENT OF [ΣP]

ION SPECIES	pH					
	4.0	5.0	6.0	7.0	8.0	9.0
$H_2PO_4^-$	98.6	99.2	93.8	60.3	13.3	1.5
$HPO_4^=$	0.06	0.65	6.1	39.2	86.7	98.5

competitively inhibits each species at its respective binding site as expressed by the following equations:

$$(12) \qquad K_{1M} = \frac{[R_a^+][H_2PO_4^-]}{[R_aH_2PO_4]}$$

$$(13) \qquad K_{1i} = \frac{[R_a^+][OH^-]}{[R_aOH]}$$

$$(14) \qquad K_{2M} = \frac{[R_b^+][HPO_4^=]}{[R_bHPO_4^-]}$$

$$(15) \qquad K_{2i} = \frac{[R_b^+][OH^-]}{[R_bOH]}$$

The conservation equations for total phosphate, $[\Sigma P]$, and the two binding sites, $[\Sigma R_a^+]$ and $[\Sigma R_b^+]$, are as follows:

$$(16) \quad [\Sigma P] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^=] + [PO_4^{\equiv}] + [R_aH_2PO_4] + [R_bHPO_4^-]$$

$$(17) \quad [\Sigma R_a^+] = [r_a^+] + [R_aOH] + [R_aH_2PO_4]$$

$$(18) \quad [\Sigma R_b^+] = [r_b^+] + [R_bOH] + [R_bHPO_4^-]$$

A cubic equation results when equations *9* through *18* are combined with the elimination of all variables except either $[R_aH_2PO_4]$ or $[R_bHPO_4^-]$, the intermediate complexes. Solution was obtained by mapping from the following partially combined equations:

$$(19) \quad [R_aH_2PO_4] = \frac{[R_bHPO_4^-][H^+](K_{2M})(K_{1i})[R_a^+]\{K_{2i} + [OH^-]\}}{(K_{1M})(K_{2i})(K_{2b})\{[R_b^+] - [R_bHPO_4^-]\}\{(K_{1i}) + [OH^-]\} + [R_bHPO_4^-][H^+](K_{2M})(K_{1i})\{(K_{2i}) + [OH^-]\}}$$

and

$$[\Sigma P] = \frac{[R_bHPO_4^-][H^+]^2(K_{2M})\{[H^+] + (K_{1h})\}\{(K_{2i}) + [OH^-]\} + [R_bHPO_4^-](K_{2M})(K_{1h})(K_{2h})\{[H^+] + (K_{3h})\}\{[OH^-] + (K_{2i})\} + [H^+](K_{2i})(K_{1h})(K_{2h})\{[R_bHPO_4^-] + [R_aH_2PO_4]\}\{[R_b^+] - [R_bHPO_4^-]\}}{[H^+](K_{2i})(K_{1h})(K_{2h})\{[R_b^+] - [R_bHPO_4^-]\}}$$

Since $[R_aH_2PO_4]$ and $[R_bHPO_4^-]$ are functions solely of the parameters $[H^+]$, $[OH^-]$, $[\Sigma P]$, $[\Sigma R_a^+]$, $[\Sigma R_b^+]$ and the several equilibrium constants, it is apparent that absorption must be described as follows:

$$(20) \quad \text{Absorption} = \{[R_aH_2PO_4](k_{a3}) + [R_bHPO_4^-](k_{b3})\}t_{sec}$$

To test the expression, values for R_a^+ and R_b^+ as well as for k_{a3} and k_{b3} must be obtained. Consideration of the results shows that only limits and approximations of these values are necessary.

Kinetic studies of enzyme reactions are usually expressed as:

$$(21) \qquad E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

where k_4 is negligible, and the rate of the observed velocity of the reaction is directly proportional to the concentration of the enzyme-substrate compound, ES, at all values of the concentration of the substrate, ΣS. Thus, $v = (ES)k_3t$ and at infinite substrate concentration the numerical constant representing the maximum velocity, V_{max}, obtained when E exists completely in the form ES, may be expressed as $V_{max} = (E)k_3t$. These considerations are valid to most enzymatic measurements, as either the appearance of product, P, the disappearance of substrate, S, or the intermediate, (ES), is determined.

Formally analogous considerations are involved in ion absorption, equations *1* and *2*. However, total ion associated with the root is measured in absorption studies. Thus, the absorption involves MR as well as M_{inside}. Absorption must then be expressed as shown in equation *22*.

$$(22) \qquad \text{Absorption} = (MR)k_3t + (MR),$$

and the maximum absorption as:

$$(23) \qquad V_{max} = (R)k_3t + (R).$$

Further, if non-specific adsorption is involved, the expression is complicated in the following manner:

$$(24) \qquad V_{max} = (R)k_3t + \{(R) + (\text{adsorption})\}.$$

A small, but measureable, amount of the sorbed phosphate is exchangeable in 30 minutes (8). It cannot be ascertained whether such exchange involves adsorbed phosphate, phosphate in combination with the carrier, or both. Inspection of equation *24* shows that if a measureable amount of adsorption contributed to the results, it would only suffice to make (R) smaller. Therefore, the highest possible value for the amount of carrier, R, may be determined by assuming a negligible non-specific adsorption according to equation *23*.

The maximum absorption, V_{max}, was determined by graphical treatment of results from three different absorption periods at $[\Sigma P]$ of 10^{-4} M to 10^{-6} M at pH 7.0, table III. Since the V_{max} for both absorption processes remain constant at varying pH values, figure 2, no restriction of the results is invoked by the selection of pH 7.0 for determining the V_{max}.

$$5.96 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,3600 + (R^+_{a+b})$$

$$8.34 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,5400 + (R^+_{a+b})$$

$$13.56 \times 10^{-7} = (R^+_{a+b})k_{3_{a+b}}\,9000 + (R^+_{a+b})$$

Solution of the three combinations of paired equations gave $(k_{3_{a+b}})$ values of 1.1×10^{-3}; 1.6×10^{-3}, and

TABLE III

MAXIMUM ABSORPTION, $(V_{a_{max}} + V_{b_{max}})$, DETERMINED FROM THREE DIFFERENT ABSORPTION PERIODS AT $[\Sigma P]$ OF 10^{-4} M TO 10^{-6} M AT PH 7.0

ABSORPTION PERIOD SEC	$(V_{a_{max}} + V_{b_{max}})$ MOLES P/GM ROOT $\times t_{sec}$
3600	5.96×10^{-7}
5400	8.34×10^{-7}
9000	13.56×10^{-7}

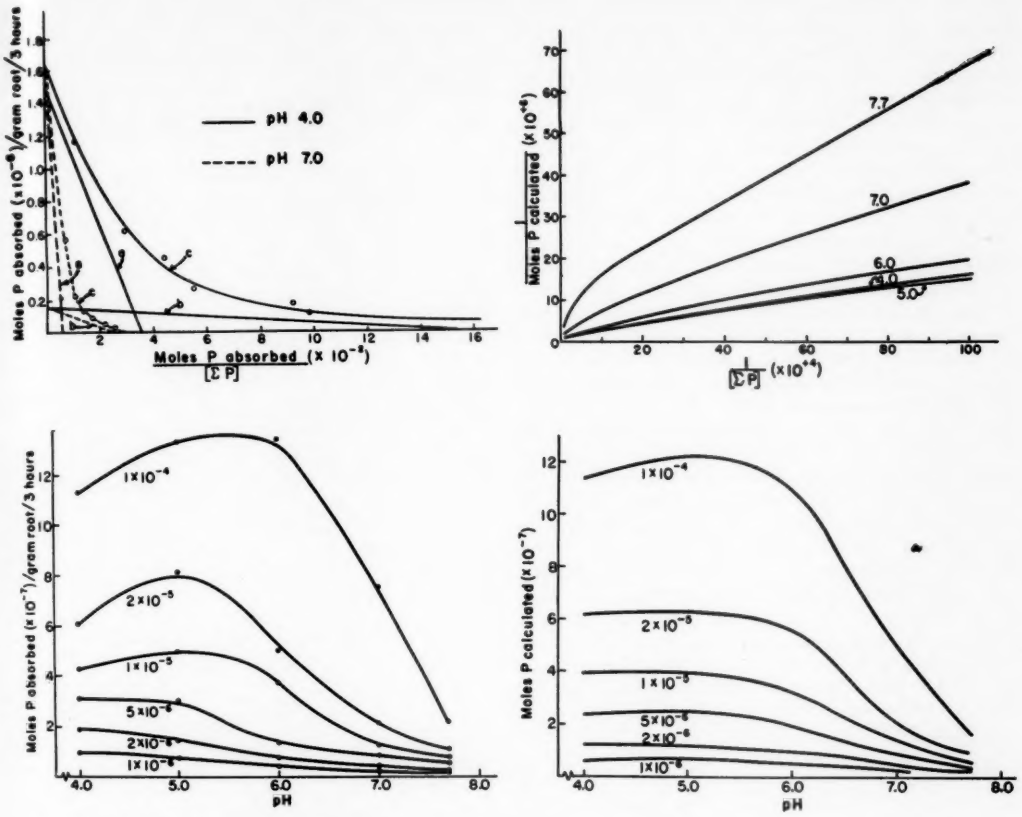


FIG. 2 to 5. Phosphate absorption by excised barley roots.

FIG. 2 (*top left*). Graphical separation of reactions, curves a and b, comprising phosphate absorption at pH 4.0 and 7.0.

FIG. 3 (*bottom left*). Absorption as a function of pH and total phosphate concentration.

FIG. 4 (*top right*). Double reciprocal plot of calculated values of phosphate absorption at varying pH.

FIG. 5 (*bottom right*). Calculated absorption as a function of pH and total phosphate concentration.

2.8×10^{-3} moles P/mole (R^+_{a+b})/gm root × sec, and from these values the mean value of (R^+_{a+b}) was calculated to be 8.72×10^{-8} moles. The average value for (R^+_{a+b}) is the greatest total amount of carrier that could be involved in absorption of both $H_2PO_4^-$ and $HPO_4^=$ from the external solution.

Absorption has been shown to be composed of two first order reactions with V_{max} for $HPO_4^=$ and $H_2PO_4^-$ absorption as 1.5×10^{-7} and 1.5×10^{-6} moles P/gm root × 3 hrs, respectively, figure 2. Thus, the $(R^+_{a+b})(k_{3_{a+b}})$ values vary by one factor of ten. To ascribe this to a difference of (R^+_{a+b}), $(k_{3_{a+b}})$, or both is not possible. Therefore, the assumption was made that equal amounts of both carriers were present, having a high value of 1×10^{-8} moles. With this value, the rate constants k_{3_a} and k_{3_b} for $HPO_4^=$ and $H_2PO_4^-$ absorption are calculated to be 1.39×10^{-3}, and 1.39×10^{-2} moles P/moles R^+/gm root × sec, respectively. A simplifying assumption, discussed in detail later in this paper, is made that the amount of carrier associated with the roots is effective throughout the volume of one liter and as such may be expressed in units of concentration. The amount of R^+ in moles is then assumed to be equivalent to moles/l of R^+. It will be shown later that the assumption of 10^{-8} M (R^+) is not critical insomuch as only $[R^+]$ greater than 10^{-6} M significantly affects the results.

The three steps in the ionization equilibria of solutions of orthophosphoric acid are: $K_{1h} = 7 \times 10^{-3}$; $K_{2h} = 6.5 \times 10^{-8}$; and $K_{3h} = 5 \times 10^{-13}$ (4).

The steady-state analysis of Michaelis and Menten is based on the assumption that the uncoupled substrate can be approximately represented by the total concentration of the substrate. This assumption is not inherent in the mathematical expression of the system, *19*, since the substrate conservation equation has been considered by *16*. However, in order to obtain the numerical values of the related constants involved in the expression, it was necessary to experi-

mentally approximate the conditions of steady-state throughout the absorption periods. Within the limits of the experimental conditions, a linear rate of phosphate absorption was shown to exist throughout an absorption period of three hours. Thus, a sufficient total amount of substrate, M, in respect to the coupled intermediate, MR, was present to maintain the concentration of M essentially constant throughout the absorption periods. This allowed determinations of constants from expressions based on the assumption of steady-state (9, 11).

The dissociation constants of the carrier phosphate intermediates, $R_aH_2PO_4$ and $R_bHPO_4^-$, can be determined only in the absence of interfering ions. Inspection of figure 1 shows that with a ten fold increase in the concentration of the competitive hydroxyl ion, in the range of pH 4.0 to 5.0, a relatively small inhibition of phosphate absorption occurs. From this, it is inferred that the effect of the competing hydroxyl ion is negligible at pH 4.0, and at this pH the dissociation constants of the phosphate intermediates may be determined. In these experiments, pH values below 4.0 were avoided because of the possibility of irreversible damage to the roots. The validity of the above reasoning will be shown in a later portion of this paper when the determination of the dissociation constants of the carrier hydroxyl ion complex is discussed.

Both processes involved in phosphate absorption at pH 4.0 are expressed by a velocity equation, *3*, derived for reactions in absence of interfering ions or competing substrates. The intercepts with the ordinate give the V_{max} directly for each reaction, when a plot is made of the variables, v against $v/[\Sigma P]$, figure 2. The V_{max} for the reactions are 1.5×10^{-6} M and 1.5×10^{-7} M, respectively. The values of the slopes give dissociation constants, K_M, of the two first order reactions in respect to the total phosphate concentration, $[\Sigma P]$. Since the dissociation constants are expressed as the equilibria between the specific ion species and its respective carrier, equations *12* and *14*, the constants must be calculated in respect to the concentration of the particular ion species under consideration. The constants describing the three ionization equilibria of orthophosphoric acid are known, and the concentration of the various species of phosphate ions can be calculated at any pH value. At pH 4.0, the $H_2PO_4^-$ and $HPO_4^=$ are approximately 98.6 % and 0.064 % of the total phosphate, respectively. Thus, the slope, K_M, of each line may be recalculated on the basis of the total concentration of the specific ion species. The numerical values for the apparent constants K_{1M} and K_{2M} were calculated to be 4.0×10^{-5} M and 5.5×10^{-10} M, respectively.

At pH 7.0 the hydroxyl ion, OH^-, interferes in a competitive manner with the absorption of both $HPO_4^=$ and $H_2PO_4^-$, figure 2. The absorption contributed by each of the two first order reactions to the total absorption may be calculated at any total substrate concentration, $[\Sigma P]$, at this pH value. The recalculation of the concentration of the specific ion involved under these conditions, pH 7.0, for each respective curve shows that $[H_2PO_4^-]$ and $[HPO_4^=]$ comprise 60.3 % and 39.2 % of the total phosphate concentration, respectively. The dissociation constant of a strictly competitive inhibitor is expressed by the following expression:

$$(25) \qquad K_i = \frac{(K_M)(I)(v)}{(V_{max})(S) - (K_M)(v) - (S)(v)}$$

As previously shown, the respective K_M and V_{max} have been determined for each of the ion species involved. The K_{1i} and K_{2i} are calculated to be 2.5×10^{-8} M and 2.0×10^{-11} M, respectively, by substitution of the respective K_M and V_{max} and the calculated concentration of the specific ion, $H_2PO_4^-$ or $HPO_4^=$ involved in its respective contribution, v, to the total absorption at a total inhibitor concentration, $[OH^-]$, of 1×10^{-7} M.

The appearance of K_M in equation *25* permits testing the validity of the observation of an insignificant inhibition of hydroxyl ion at pH 4.0. Using the value of K_M based on this observation, the values of K_{1i} and K_{2i} were calculated from pH values 6.0, 7.0 or 7.7, respectively, and shown to remain essentially constant. A significant error in the numerical value of K_M due to hydroxyl ion competition at pH 4.0 would be reflected by varying values of K_{1i} and K_{2i} when calculated from the results obtained at the different pH values.

Agreement with Experimental Observations

Substitution of the apparent constants into equation *19* permitted values for the intermediates, $[R_aH_2PO_4]$ and $[R_bHPO_4^-]$, at varying $[\Sigma P]$ to be calculated for each pH level. Since the concentration of each intermediate complex is assumed to be equivalent to the amount in moles, the values were multiplied by the respective k_3t according to equation *20*, where t is equal to 10,800 seconds. A double reciprocal plot of the calculated total absorption against $[\Sigma P]$ is shown in figure 4. Calculated total absorption at different pH values is shown in figure 5. Essential agreement with experimental results, figures 1 and 3, indicates that the expression describes the system for phosphate absorption by excised barley roots.

Discussion

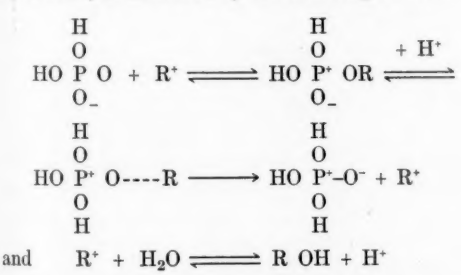
Association of the binding sites involved in phosphate absorption with roots describes a heterogeneous system. Although the general kinetic treatment employed in this paper is applicable to both homogeneous and heterogeneous reactions, certain restrictions are imposed on the results obtained from heterogeneous systems.

Consideration of equilibrium in intermediate complex formation necessitates assignment of concentration values for the intermediate complexes, $R_aH_2PO_4$ and $R_bHPO_4^-$, and the binding sites, R_a^+ and R_b^+. The units of concentration are expressed in respect to the external system and do not indicate the concentration of the sites in the roots, per se. Since the absolute concentrations of the binding sites are not known, it is evident that constants describing reactions with

these sites must be recognized as apparent or relative constants. Under the experimental conditions, it has been shown that the greatest amount of $(R_a^+ + R_b^+)$ effective in phosphate absorption from the external solution is approximately 1×10^{-8} moles. The measured maximum absorption for each reaction is expressed by an $(Rk_3t + R)$ which dictates that varying amounts of R^+ are compensated by changing values of k_3. Since the moles of R^+ have been assumed to be equivalent to the concentration of R^+ in respect to the external system, it can be shown that only $[R^+]$ of 1×10^{-6} M or greater significantly affect the equilibrium as described by equation *19*. The assignment of the highest value to the concentration of the binding sites is not critical to this analysis since $[R^+]$ is 1×10^{-8} M, or less.

Phosphate absorption by barley roots emphasizes the danger in assigning importance to an ion solely because of its predominant concentration in the system. The first step in the ionization equilibria of orthophosphoric acid differs from the second step by approximately five factors of ten, being 7×10^{-3} M and 6.5×10^{-8} M for K_{1h} and K_{2h}, respectively. Consideration of concentration of the ions, per se, would dictate that $HPO_4^=$ is unimportant in the absorption of phosphate at acid pH values, since the ionization of $H_2PO_4^-$ to $HPO_4^=$ is small. However, the apparent dissociation constants of the two phosphate intermediates differ by five factors of ten; the K_{1M} and K_{2M} being 4×10^{-5} M and 5.5×10^{-10} M, respectively. Since total phosphate has been shown to be greatly in excess to that of the binding groups, $(R_a^+ + R_b^+)$, and the ionization of orthophosphate is not rate limiting, it is apparent that the $HPO_4^=$ ion, in less prominent concentration but with greater affinity, attains importance in absorption equal in magnitude with that of the $H_2PO_4^-$.

The competitive nature of the hydroxyl ion, OH^-, indicates the formation and breakdown of the labile, intermediate complexes involves the cleavage of an R–O bond, as described by the following reactions:



and $R^+ + H_2O \rightleftharpoons R\,OH + H^+$

If the P–O bond were involved, a hydrogen ion competition would be expected, which is not the case, figure 2. Cleavage of such $R\text{–}OPO_3$ bonds is known to be involved in such reactions as the acid hydrolysis of glucose-1-phosphate (1) and numerous examples of phosphorylase action on glucosyl–OPO_3 (1, 5).

Summary

Kinetics of phosphate absorption by excised roots of *Hordeum vulgare* var. Atlas 46 were studied in constantly aerated solutions at 30° C with phosphate containing P^{32}. In order that concentrations might remain essentially constant during three hour sorption periods one liter quantities of solutions were used with one gram of roots. Principle variables were phosphate (10^{-4} to 10^{-6} M) and hydrogen ion (pH 4 to 8) concentration. A quantitative interpretation of the results was found. This interpretation is based on the formation and breakdown of phosphate complexes with metabolically derived reactants (sites).

(1) In excised barley roots $H_2PO_4^-$ is absorbed through one site and $HPO_4^=$ through another.

(2) Hydroxyl ion competitively inhibits absorption of both $H_2PO_4^-$ and $HPO_4^=$.

(3) Neither hydrogen nor hydroxyl ions in the external medium affects the concentrations of either absorption site effective in phosphate absorption from the external solution.

(4) The rate limiting breakdown of both kinds of intermediate phosphate compounds involves cleavage of an R–O bond.

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STUDIES ON THE MECHANISM OF PHYTOHORMONE DAMAGE BY IONIZING RADIATION. I. THE RADIOSENSITIVITY OF INDOLEACETIC ACID[1]

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The phytohormone auxin is widely recognized as a regulator of growth and morphogenesis. Since retarded growth and altered developmental patterns in plants are known to follow exposure to ionizing radiation, Skoog (26) investigated the inactivation of auxin by x- and γ-rays. His data indicated that both native auxin preparations and pure 3-indoleacetic acid (IAA) in solution were highly labile when irradiated. A marked reduction in auxin levels also occurred when plant tissues were given moderate doses of x-radiation. Auxin thus appeared to be radiosensitive in vitro and in vivo. Skoog pointed out the parallel effects of decreased auxin levels and low doses of radiation in growth and correlation phenomena. His results were used subsequently by other workers to interpret various radiation responses on the basis of auxin radiosensitivity (10, 16, 27).

However, certain considerations discussed below cast doubt on the implied direct sensitivity of the auxin molecule, and prompted our reinvestigation of auxin radiosensitivity. Concomitantly, a more precise evaluation of IAA inactivation kinetics was obtained. The present work deals with the destruction of auxin in aqueous solution by ionizing radiation. Auxin sensitivity in the plant, and the biochemical mechanisms whereby auxin levels are lowered in vivo, will be treated in subsequent papers of this series.

PRELIMINARY CONSIDERATIONS

The loss of auxin in the irridated plant was attributed wholly or in part to direct inactivation (26). A direct auxin photolysis seems remarkable in view of the low auxin concentrations relative to other, potentially protective, cellular components in plant tissues. It is likewise striking that irradiation of both in vivo and in vitro systems with comparable doses resulted in similar percent inactivations. In order to approximate the kinetics and efficiencies of auxin inactivation in vitro, we combined the data from Skoog's (26) tables 2 and 4, and figure 1. In the range of 0 to 4,000 roentgens, these data could be characterized satisfactorily by the relationship

$$C_r = A_1 e^{-b_1 r} + A_2 e^{-b_2 r} \qquad (1)$$

where C_r is the auxin concentration in μ moles/ml at any roentgen dose, r. A_1, A_2, b_1, b_2 are constants > 0, and $A_1 + A_2 =$ the intial auxin concentration, C_o. With $C_o = 0.015$ μ moles/ml, graphical fit yielded the following values of the constants: $A_1 = 0.005$, $b_1 = 0.041$, $A_2 = 0.01$, $b_2 = 0.00011$. For later comparison, the fitted curve of C_r versus dosage is given in figure 1, curve A.

The chemical effects of ionizing radiation on dilute aqueous solutions are attributed chiefly to free radicals formed through excitation and ionization of water (6, 17). For a known dose of a given radiation energy, the number of primary ion pairs formed per unit weight of water can be calculated, and the efficiency of solute inactivation conveniently expressed as ionic yield (M/N), i.e., the number of solute molecules inactivated (M) per ion pair (N). Under the conditions of continuous uniform irradiation and nonlimiting solute concentrations, with no competition for the radicals by solute inactivation products, and with back reactions at a steady-state, the concentration of solute decreases linearly with the dose (17). The ionic yield would thus remain constant. Where varying rates of back reactions, competition, or a chain inactivation occurs, residual solute concentration will diminish either exponentially or at rates which can be described empirically by sums of exponentials. In these instances M/N will vary with increasing dosage. Under such circumstances an estimate of the intrinsic radiosensitivity of the solute could be obtained by determining the inactivation efficiency at *initiation* of irradiation, i.e., as the dose approaches zero. This initial ionic yield with exponential inactivations may be estimated by calculating the M/N for the various experimental values and either approximating graphically the ionic yield at zero dose or evaluating empirically the relationship $\lim_{r \to o} M/N = (f)r$.

A convenient and more accurate approximation may be made of the initial ionic yield when M/N vs r is nonlinear. Ionic yield for any roentgen dose may be expressed as

$$(M/N)_r = \left[\frac{C_o - C_r}{r}\right] K \qquad (2)$$

where K is the quotient of Avogadro's number divided by the number of ion pairs formed per r per unit volume, and C_o, the initial solute concentration, and C_r, the concentration remaining at dose r, are in moles per unit volume.

Let

$$C_r = \sum_{i=1}^{n} A_i e^{-b_i r} \qquad (3)$$

where the A_i's and b_i's are constants > 0, and

$$\sum_{i=1}^{n} A_i = C_o.$$

Then

[1] Received September 23, 1954.

$$(M/N)_r = \frac{\left[C_0 - \sum_{i=1}^{n} A_i e^{-b_i r}\right] K}{r} \quad (4)$$

Expanding the exponential terms, and recalling

$$\sum_{i=1}^{n} A_i = C_o,$$

$$(M/N)_r = K \sum A_i b_i \left[1 - \frac{b_i r}{2!} + \frac{b_i^2 r^2}{3!} - \ldots\right]. \quad (5)$$

Hence

$$(M/N)_{r\to 0} = K \sum_{i=1}^{n} A_i b_i \quad (6)$$

For n = 1,

$$(M/N)_{r \to o} = K\, A_1 b_1$$

For n = 2,

$$(M/N)_{r \to o} = K(A_1 b_1 + A_2 b_2) \text{ etc.}$$

If we assume an effective wavelength of 0.02 Å in Skoog's experiments (heavily filtered 900 kvp x-rays), and 1.8×10^{12} ionizations per ml solution for this wavelength (17), K becomes 2.4×10^{11}. By inserting the constants of equation (*1*) in equation (*6*), n = 2, an initial ionic yield of ca 70 is obtained. An even higher intrinsic sensitivity is shown in the inactivation of auxin by gamma radiation. A fit of the data obtained by irradiating auxin solutions with a radium source (26, p. 236) gave residual concentrations the values of

$$C_r = 0.0027\, e^{-0.35r} + 0.012\, e^{-0.0009r} \mu \text{ moles/ml} \quad (7)$$

From equation (*6*) an initial ionic yield of 325 results; the curve obtained by exponential fit of the ionic yields calculated for each experimental point yielded an initial inactivation rate of 350 molecules inactivated per ion pair.

These ionic yields are unusually high. Many biological substances are inactivated by ionizing radiation with efficiencies on the order of a few hundredths to somewhat above 1 molecule per ion pair (17). Ionic yields somewhat greater than 1 have been interpreted as resulting from the production of several active radicals after the primary splitting of water to H and OH (4, 14). However, yields of 70 and 325 indicate that a chain or autocatalytic sequence of auxin inactivation had been induced, similar to the radiation induced auto-oxidation of unsaturated fatty acids (19, 22) or the decomposition of indole with very low dosages (Allsopp in (6)).

The difference in the initial inactivation efficiency of very hard x- and γ-irradiation is difficult to interpret. The number of ionizations produced per ml solution per r, as well as the linear ion density in this instance, are similar for both radiations. One would expect the radiochemical effects to be essentially the same, as they have been found to be with even wider ranges in energies (17, 21). Therefore, we first attempted to duplicate the above radiochemical efficiencies for auxin inactivation.

Experimental Methods

Stock solutions of IAA (Eastman) in purified water (13), generally 0.25 mM, were made up monthly and stored at 3 to 5° C. Lower concentrations were freshly prepared from stock solution. To obtain higher concentrations, the more soluble Na salt (recrystallized from the acid) was utilized. Unless otherwise noted, the solutions were in equilibrium with air and irradiated at a pH of 6 to 7. Solutions with low oxygen concentration were prepared by the evacuation apparatus and method described by Hart (13). The same apparatus was used to saturate solutions with oxygen (15).

IAA was usually assayed colorimetrically with iron-perchloric acid reagent (11). In testing the protective action of other solutes, IAA calibration curves were determined in the presence of the protective agent in amounts equivalent to those experimentally used. When bioassay was used to measure auxin concentration, diluted aliquots of a solution were mixed with an equal quantity of 3 % liquid agar and assayed by the standard Avena curvature method using sand-grown plants. Simultaneously, several known concentrations of IAA were run in order to convert experimental curvatures directly to IAA mole equivalents.

Native auxin of the kidney bean plant was extracted with ether and purified by paper chromatography. Several kg of 3-week-old plants, cut at the soil level, were frozen in liquid N_2 and pulverized. The material was covered with diethyl ether (distilled from aqueous $Ca(OH)_2$-$FeSO_4$) which was allowed to remain at 0 to 3° C for ½ hour and then decanted. This extraction was twice repeated. The combined extracts were reduced in volume to ca 100 ml and partitioned 3 times with 20 ml of 1 % $NaHCO_3$ in water. The combined aqueous phases were washed once with ether, adjusted to pH 3.0 with HCl, and repartitioned with ether (3 × 2 vols). After removal of the ether from the acid fraction by distillation, the residue was taken up in 0.5 ml of purified 95 % ethanol (distilled from Zn-KOH). The alcohol was then quantitatively transferred and evaporated in a nitrogen stream as the starting line of a chromatogram (6.25 cm strip Whatman No. 3 MM). Following equilibration in the chromatograph chamber, the strip was developed by descending isopropanol : 28 % ammonia : water (10 : 1 : 1) at 25° C for 16 hours.

The following radiation sources were employed:

1. For hard x-rays,
   a. Westinghouse 200 kvp, filtration 0.75 mm Cu plus 3 mm Bakelite, half-value layer 1.07 mm Cu, λ effective 0.15 Å.
   b. G. E. Maximar 250 kvp, filtration 0.25 mm Cu plus 1.5 mm Bakelite, hvl 1 mm Cu, λ effective 0.15 Å.
2. For soft x-rays, Picker 50 kvp, no added filtration, hvl 1.07 mm Be or 0.07 mm Al, λ effective approximated as 1.8 Å.

3. For γ-rays,
   a. 100 mg radium needle in equilibrium with its decay products, whose λ effective was considered as 0.015 Å on the basis of the average total photon energy (5).
   b. The 25 curie Co^{60} source described in (13).

Unless otherwise noted, the dose rates were between 50 and 250r/min for x-rays, 12.2r/min with the Ra source, and 440r/min with the Co^{60} source. (As will be shown below, there is no appreciable effect of dose rate on the ionic yield in this range.)

In a large fraction of the experiments with hard x-rays, the solutions were in equilibrium with air and irradiated in 50- or 100-ml silica glass cylinders under a vertical beam. Since the heights of the solutions were 2.5 to 5 cm, the filtered hard x-rays were considered as monochromatic and as decreasing exponentially through the water. Victoreen thimble chamber measurements of dose rate were made at the aqueous surface of a phantom and converted to integral dose by Mayneord's approximation (18, equation (7)), using 0.181 as the absorption coefficient for water at λ 0.15 Å (7). In a typical vertical irradiation of a cylindrical volume of solution by the 200 kv x-ray beam, target to surface distance 25 cm, the Mayneord approximation gave an average dose rate of 85r per minute. A depth-dose plot (8) for the volume yielded 83r, while a determination by ferrous ion oxidation, under identical irradiation conditions, gave a value of 73r. Radiation dose was determined in all other experiments by ferrous sulfate oxidation, using the value of 19.7 μmoles of Fe^{++} oxidized per liter per kiloroentgen to convert the iron yield to r (20, 21). The experimental values given for radiation inactivation are corrected for similarly treated but unirradiated controls.

Results

A 15.5 μM solution of cryst. IAA in air-saturated water was irradiated with hard x-rays. The changes in residual auxin concentration were followed colorimetrically, and are shown in figure 1, curve B. The concentration decreased exponentially,

$$C_r = .0155\ e^{-0.000158r}\ \mu\text{moles/ml} \qquad (8)$$

from which, using equation (6), an initial ionic yield of 0.87 is obtained. These results may be contrasted with those in (26) as differing both in rate order of inactivation and in direct molecular radiosensitivity.

Method of Auxin Assay: Two changes from the procedures used in (26) were made in the above experiment. First, the concentration of auxin involved permitted use of the more rapid and convenient colorimetric assay instead of biological assay as a measure of changes in IAA concentration. If we assume that the products arising from IAA on irradiation are of reduced activity or are relatively inactive as auxins when measured by coleoptile curvature—certainly a reasonable assumption in view of the postulated structural requirements for biological activity—it is still possible that such products could react with the color reagent. Colorimetrically determined values might, therefore, be spuriously high. This possibility is ruled out by the curves later shown in figure 7. Concomitant analyses by both assay methods of aliquots taken from a solution of IAA during irradiation yielded essentially the same values.

Radiation Energy: Second, since we used "hard" (not "very hard") x-rays, it was possible that the kinetics of auxin inactivation were uniquely dependent on photon energy in this frequency range. Though the probability of such a dependence was low, as indicated above, the inactivation efficiencies of gamma and hard and soft x-rays were examined. Figure 2 shows the IAA concentrations during irradiation of 53.6 μM solution by radium and by 200-kv and 50-kv x-rays. It is evident that the inactivation curves are similar for the three radiations, the initial ionic yields being about 1. In an analogous experiment, an 860 μM solution was irradiated with the Co^{60} source. An initial ionic yield of about 1.2 was obtained.

Auxin Concentration: A series of indoleacetate concentrations was then irradiated by hard x-rays. The solutions were unbuffered (pH 6 to 7), and ranged in concentration from 15 to 1200 μM. Residual IAA concentrations at the various doses for seven typical experiments are shown in figure 3. The exponential form is apparently followed irrespective of initial concentration or dose. With the three lowest concentrations, the exponential relation holds till approximately 90 % of the auxin originally present was inactivated.

The residual concentration curves in figure 3 can be generalized as

$$C_r = C_o\ e^{-br}$$

with the initial condition $C_r = C_o$ when $r = o$. It is evident that the slopes of the linear relationships log C_r versus dosage are not equal but vary inversely with C_o. Therefore, let

$$b = \frac{k}{C_o}, \text{ whence}$$

$$C_r = C_o\ e^{-kr/C_o} \qquad (9)$$

$$C_o - C_r = C_o\ (1 - e^{-kr/C_o}) \qquad (10)$$

The experimental data permitted a test of the preceding generalizations. A log-log plot of b versus C_o for nineteen irradiation experiments, in which each slope was approximated by least squares, is given in figure 4. A least squares fit yields the value for b of $3.7 \times 10^{-6}\ C_o^{-0.90 \pm 0.034}$, with C_o expressed in μmoles/ml. Assuming that the exponent of C_o is -1, the intercept value of k for equation (9) is 2.8×10^{-6} (fig 4). If the initial concentrations below 0.05 μmoles/ml, where the yield tends to fall, are not considered, $b = 3.1 \times 10^{-6}\ C_o^{-1.03}$. Equations (9) and (10) may therefore be considered as reasonably correct formulations of auxin inactivation by hard x-rays within the concentration and dose range studied.

We have no specific knowledge of the number, identity, concentrations, or reactivities of the auxin inactivation products formed during the irradiation.

However, the concentration of such products may be expressed in terms of the IAA inactivated, $C_o - C_r$, having the average radical reaction affinity relative to IAA of β. Per unit volume the increment of IAA molecules inactivated on any dose increment will depend on the ratio of IAA to the total number of reactive molecules and their reaction affinities. If we assume that the only solutes present in pure solution are auxin and its inactivation products, and that solute concentrations are sufficiently high so that we may neglect the probability of radical elimination by activated water interactions, then:

$$dC_r = -kC_r dr\ [C_r + \beta(C_o - C_r)]^{-1}. \qquad (11)$$

This approximation may be integrated to

$$C_r(1-\beta) + \beta C_o \ln C_r = -kr + A \qquad (12)$$

For $\beta = 1$,

$$\ln C_r = -kr/C_o + A/C_o$$

$$C_r = A' e^{-kr/C_o} \qquad (13)$$

For the initial conditions $C_r = C_o = A'$ when $r = o$, equations (*9*) and (*13*) are identical. We may therefore infer that the kinetics of IAA destruction in pure solution are defined in part by the competitive action of the inactivation products, and that such products have an average reaction affinity for the activated water about equal to that of IAA. A like reaction order was found in the x-ray inactivation of carboxypeptidase by Dale et al (3). There, similarly, the proportion of active enzyme concentration remaining at various doses was a function of the ratio of the dose to initial enzyme concentration over two orders of magnitude of C_o.

Finally, the initial ionic yields were calculated for each of the above irradiations with various auxin concentrations. They are shown in figure 5, curve A. Within two orders of concentration magnitude, the ionic yields are essentially in the range of unity. There is a trend toward lower initial ionic yields at higher dilutions. This is consistent with previous observations (17), in which falling radiochemical yields at high solute dilutions are attributed to an increased probability of radical recombination before reaction with the solute. The fall in yield at higher dilutions, as well as the relatively constant absolute amount of auxin inactivated for a given dose over the major portion of the concentration range, is approximated by equation (*10*).

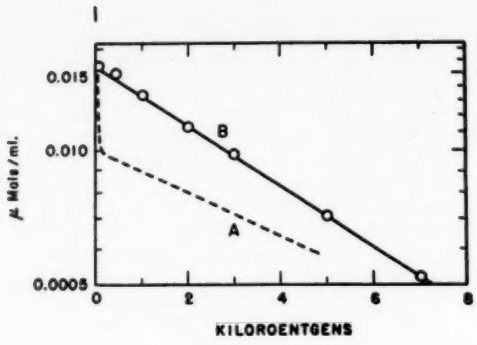
OXYGEN: The presence of dissolved oxygen generally enhances the oxidation of a solute by x- or γ-rays. We therefore considered the possibility that the efficiency of auxin inactivation might be critically dependent upon oxygen concentration in the solutions. Various concentrations of auxin in water were either evacuated or saturated with oxygen and then exposed to hard x-rays. The initial ionic yields calculated for each auxin concentration are given in figure 5. In comparison with the ionic yields of about 1 for auxin solutions in equilibrium with air, the yields dropped to between 0.5 and 0.3 in the absence of air and were slightly above 1 with oxygen saturation. In no instance was there any indication of initiation of a chain sequence of auxin inactivation or highly accelerated inactivation rates.

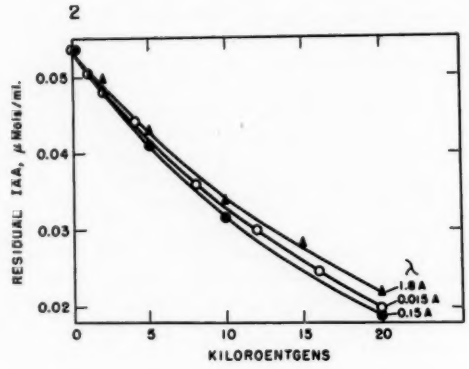
PH: The statement that the extent of auxin inactivation in aqueous solution (in presence of air) is at least as great in the pH range well above the pK of the acid as in the range below it (26), is not substantiated by our observations. When IAA solutions adjusted to various pH's by HCl or NaOH were irradiated, the extent of inactivation increased with increasing acidity (fig 6, curve A). The difference in auxin sensitivities at neutral and acid pH's was enhanced when the medium was buffered (fig 6, curves B and D). Indeed, the inactivation curves in buffer containing dissolved air bear a striking resemblance to the dissociation curve of IAA, which has a pK 4.75. This resemblance suggested that the radiosensitivity was a function of the concentration of associated molecules, a possibility considered by Skoog in examining the pH effect. However, when evacuated solutions were irradiated, no significant difference was found in the amount of inactivation between pH 2 and pH 8 (fig. 6, curve C). Since IAA dissociation equilibria presumably are unaffected by evacuation, the apparent higher sensitivity of the associated form of IAA may be related to the particular radical species produced by irradiating aerated water. Alternatively, oxidant concentration may increase with H^+ concentration when water containing dissolved O_2 is irradiated.

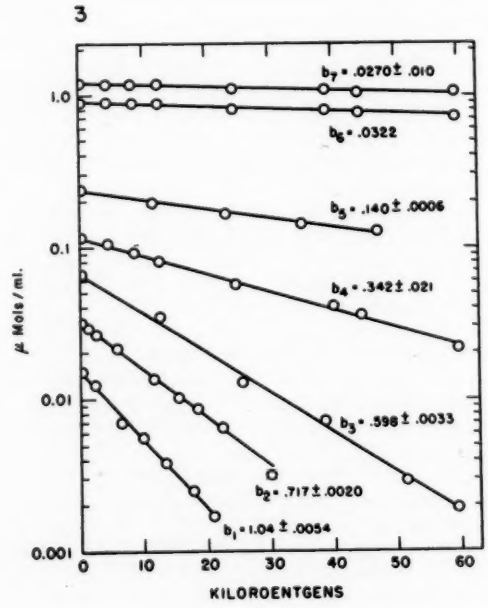
It seems unlikely that photolysis of the HCl used for acidification caused the pH effect in solutions without added buffer (fig 6, curve A); a similar difference in the amount of IAA inactivated was observed for solutions adjusted to pH 5 and pH 2 with H_2SO_4 before irradiation. Competition for radicals by buffer citrate may have caused the decreased IAA inactivation near neutrality (fig 6, curves B, C, and D). Yet, IAA inactivation increased at the more acid pH's at which citrate concentrations were also greater. Though the mechanism of yield dependencies on pH is not clear (Amphlett in (6)), it is pertinent that H_2O_2 levels in irradiated water were found to increase with increasing acidity (1).

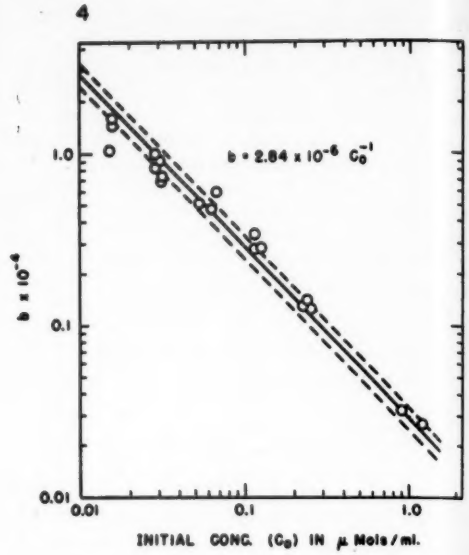
As an index of inactivation efficiency in acid medium, IAA solutions at pH 2.9 were then x-irradiated. The typical exponential decrease in concentration was observed, and, as indicated in table I, no greatly enhanced inactivation efficiency resulted at the more acid pH. The initial ionic yields are roughly double those observed on irradiation of neutral solutions.

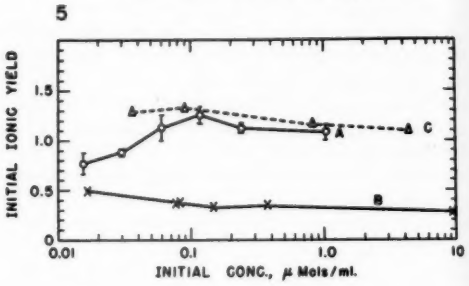
PROTECTIVE EFFECTS: With ionizing radiation, one would anticipate that the ionic yield for auxin inactivation would be decreased by the presence of other solute molecules able to react with activated water. Such protective effects have been frequently observed and investigated. Nevertheless, it is possible that enhanced inactivation might be caused by impurities present in an indoleacetate preparation or by certain cosolutes. Therefore, the inactivation of IAA by hard x-rays was determined after IAA recrystallization, in

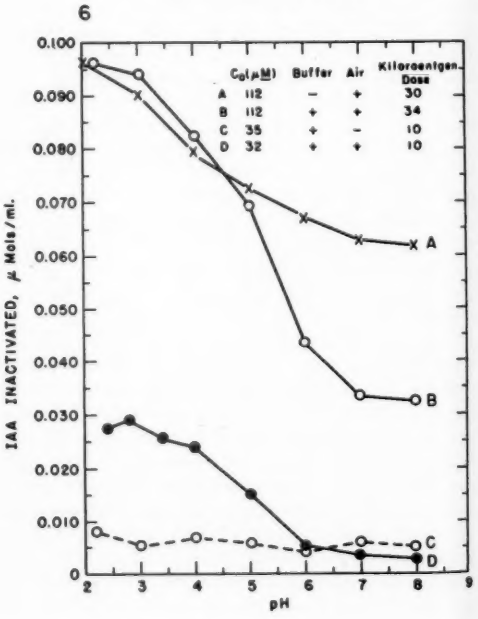

1
0.015
0.010
0.0005
μ Mols/ml.
B
A
0
2
4
6
8
KILOROENTGENS


2
RESIDUAL IAA, μ Mols/ml.
0.05
0.04
0.03
0.02
λ
1.8 A
0.015 A
0.15 A
0
5
10
15
20
KILOROENTGENS


3
μ Mols/ml.
1.0
0.1
0.01
0.001
b_7 = .0270 ± .010
b_6 = .0322
b_5 = .140 ± .0006
b_4 = .342 ± .021
b_3 = .598 ± .0033
b_2 = .717 ± .0020
b_1 = 1.04 ± .0054
0
10
20
30
40
50
60
KILOROENTGENS


4
$b = 2.84 \times 10^{-6}\ C_0^{-1}$
b x 10⁻⁴
1.0
0.1
0.01
0.01
0.1
1.0
INITIAL CONC. (C_0) IN μ Mols/ml.


5
INITIAL IONIC YIELD
1.5
1.0
0.5
0
A
B
C
0.01
0.1
1.0
10
INITIAL CONC., μ Mols/ml.


6
IAA INACTIVATED, μ Mols/ml.
0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0
C_0(μM) Buffer Air Kiloroentgen Dose
A 112 – + 30
B 112 + + 34
C 35 + – 10
D 32 + + 10
A
B
C
D
2
3
4
5
6
7
8
9
pH

the presence of single added substances, and in the presence of extracts of the Avena coleoptile and cocklebur plant.

Commercial IAA was recrystallized four times (as the Na salt from alcohol, again as the salt from water-alcohol, as the acid from water and from ethylene dichloride). The inactivation curves, given in figure 7, indicate no significant effect of recrystallization upon IAA sensitivity, the initial ionic yields being approximately one.

Inactivation curves were then determined for IAA in the presence of added solutes at several mole ratios. Irradiation and periodic sampling were continued (with the single solute added as well as with the plant extracts) till 10 to 20 % of the IAA initially present was inactivated. Initial inactivation efficiencies were calculated, and are given in table II, column 4, in order of increasing protective action. No great enhancement of IAA inactivation was observed with any of the substances listed. Glutathione, cysteine, asparagine, glucose, and ascorbate, in equimolar ratios, showed no protective effect under these experimental conditions. Significantly reduced IAA inactivation rates resulted with the last two compounds when present in 10 mole excess, and in the presence of ethanol, ferricyanide, and malic acid. It should be noted, as a potential model for in vivo auxin sensitivity, that protein protected the IAA with relatively high efficiency. The presence of the albumin in 0.1 molar ratio reduced initial IAA inactivation efficiency by ca 2/3, whereas the leaf protein, at a molar ratio of 10^{-2}, lowered the ionic yield 100 fold.

Lea (17) has formulated the number of solute molecules undergoing chemical change by radical interaction, in a unit volume, as n CZpP, where n is the number of active radicals, C is the solute concentration, Z the solute-radical collision frequency for unit solute concentration, p the probability of radical elimination on collision with the solute, and P the proportion of those radical eliminations which results in solute change. By a procedure analogous to that followed by Lea in evaluating the data of Dale (2), in which the concentrations of various cosolutes required for 50 % protection of flavin-adenine-dinucleotide against a single x-ray dose were listed, the relative radical deactivation probabilities on solute collision may be deduced for the experiments reported here. Let us consider the cosolute (C_2) as competing with

TABLE I

IONIC YIELDS BY HARD X-IRRADIATION OF INDOLEACETIC ACID AT PH 2.9, MCILVAINE'S CITRATE-PHOSPHATE BUFFERS

	C_o	b	$(M/N)_{r \to o}$
	Moles $\times 10^{-6}/ml$	$\times 10^{-4}$	
Buffered	0.126	0.57	2.5
Unbuffered	0.111	0.53	2.1
Buffered	0.032	1.95	2.2

$C_r = C_o \cdot e^{-br}$; $(M/N)_{r \to o} = C_o \cdot b \cdot K$; $K = 3.52 \times 10^{11}$ (see text)

IAA (C_1) for radicals produced from the water, and assume that a steady state of free radical concentration is rapidly established (13) under a constant dose rate. Using the above symbols, radical (R) production and radical and solute deactivation may be characterized by the following rate constants:

$$H_2O \xrightarrow{k_r} R$$

$$C_1 + R \xrightarrow{Z_1 p_1 P_1} C_1R$$

$$C_2 + R \xrightarrow{Z_2 p_2 P_2} C_2R$$

In the steady state

$$\frac{dR}{dt} = 0$$

$$R = \frac{k_r}{C_1Z_1p_1 + C_2Z_2p_2}$$

$$-\frac{dC_1}{dt} = \frac{Z_1p_1P_1C_1k_r}{C_1Z_1p_1 + C_2Z_2p_2} \qquad (14)$$

If $C_2 = 0$

$$-\frac{dC_1}{dt} = P_1k_r \qquad (15)$$

Let $a = \frac{dC_1 \text{ in presence of } C_2}{dC_1 \text{ in pure solution}}$, where $0 < a < 1$.

Then $\frac{Z_1p_1P_1C_1k_r}{C_1Z_1p_1 + C_2Z_2p_2} = aP_1k_r$, whence the relative probability of radical deactivation

$$\frac{p_2}{p_1} = \frac{Z_1C_1(1 - a)}{aZ_2C_2} \qquad (16)$$

[Equation (14) may be considered as a more explicit

FIG. 1. Residual concentrations of IAA in water after various dosages of x-rays. Initial concentrations 15 μM. Curve A. Calculated from data of Skoog, 900 kvp, 50r/min. Curve B. This work, 200 kvp, 50r/min.

FIG. 2. The inactivation of auxin by radium γ, 200-kvp and 50-kvp x-rays. The initial ionic yields are, respectively, 0.89, 0.97, and 0.84.

FIG. 3. Residual concentrations of auxin as a function of hard x-ray dose at various initial IAA concentrations. The fitted slopes are listed and all are $\times 10^{-4}$.

FIG. 4. Test of equations (9) and (10), with slopes of residual concentration curves as a function of hard x-ray dose plotted against initial auxin concentration. Broken lines indicate 99 % confidence limits from fit using C_o^{-1}.

FIG. 5. Initial ionic yields obtained by hard x-irradiation of various concentrations of indoleacetate in water. Solutions unbuffered, pH 6–7. Curve A. Solutions in equilibrium with air. Vertical brackets indicate the standard error. Curve B. Evacuated. Curve C. Oxygen saturated.

FIG. 6. The effect of pH on IAA inactivation by hard x-rays. Buffer: McIlvaine's standard citrate-phosphate.

TABLE II

EFFECT OF ADDED SOLUTES ON INITIAL IONIC YIELDS FOR IAA INACTIVATION BY HARD X-RAYS

1 SOLUTES	2 INITIAL IAA CONC	3 MOL RATIO ADDEND/IAA	4 $(M/N)_{r\to 0}$	5 p_2/p_1 †	6 Zp/MOL WT ††	7 Zp ‡	8 Z_2p_2/Z_1p_1
	μM						
Glutathione	59	1	1.0	....	....	....	....
Glutathione	63	10	2.3	....	....	....	....
Cysteine	65	1	1.3	....	....	....	....
Cysteine	61	10	1.6	....	....	....	....
Asparagine	63	1	1.2	....	....	....	....
Asparagine	62	10	1.2	....	....	....	....
Glucose	65	1	1.2	....	....	....	....
Glucose	62	10	0.57	0.11	2.1×10^{8}	3.7×10^{10}	0.11
Ascorbic acid	62	1	1.2	....	....	....	....
Ascorbic acid	62	10	0.41	0.19	3.8×10^{8}	6.6×10^{10}	0.19
Ethanol	62	1	0.98	0.33	1.6×10^{9}	7.3×10^{10}	0.21
Ethanol	62	10	0.46	0.25	1.2×10^{9}	5.5×10^{10}	0.16
$K_3Fe(CN)_6$	60	1	0.66	0.59	8.5×10^{8}	2.8×10^{11}	0.82
Malic acid	62	1	0.51	1.5	3.5×10^{9}	4.8×10^{11}	1.4
Bovine serum albumin *	62	0.1	0.37	0.81	1.1×10^{8}	7.6×10^{12}	22
Leaf protein ** "Fraction I"	61	0.01	0.011	110	6.3×10^{9}	3.8×10^{15}	11,000
IAA alone	62	....	1.2	1	1.9×10^{9}	3.4×10^{11}	1
IAA alone	21	....	0.88	....	....	....	....
IAA in ½ strength coleoptile juice	21	....	0.016	....	....	....	....

* Armour cryst., molecular wt 70,000.

** Prepared from spinach leaves according to (28), incorporating two cycles of differential centrifugation, and presumed to be over 95 % pure, molecular wt 6×10^{6}.

† Relative probability of radical elimination on single collision with a solute molecule.

†† Relative radical affinity of solutions containing 1 gm/l.

‡ Relative radical affinity of solutions containing 1 gm mole/l.

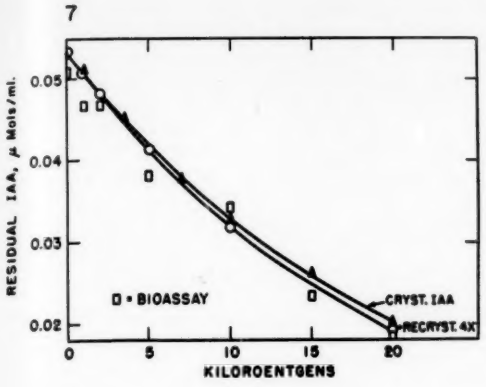
Z represents the number of collisions per sec per ml between solute molecules and an active radical calculated by the relationship $Z_{AB} = n_A n_B \delta^2_{AB}[8\pi RT(1/m_A + 1/m_B)]^{1/2}$, where n = the number of molecules per ml, m the molecular wt, δ_{AB} the mean molecular diam in cm, and R the gas constant in ergs per mole per degree (9). The molecular diameters have been approximated as 1.33×10^{-8} $m^{1/3}$ cm (17).

form of equation (*11*) in the case when C_2 represents not a cosolute added to the IAA solution, but the product(s) of IAA inactivation, whose concentration was defined as $(C_o - C_1)$. Thus, when the relative radical reaction affinity of the product is 1, i.e., $Z_1p_1 = Z_2p_2$, equation (*9*) likewise follows.]

The relative probabilities of radical elimination with a number of the cosolutes used are given in table II, column 5. It may be seen that the probability of radical deactivation with a single collision is less for low molecular weight cosolute molecules (except for malic acid) than for IAA. The probability for the serum albumin molecule is, surprisingly, likewise less, though the spinach leaf protein has an effectiveness of from 10^2 to 10^3 greater than the other substances evaluated. Since the collision *frequency* rises with increase in solute molecule dimension, the proteins have, on an equimolar basis, an appreciably greater net radical affinity than has IAA; thus they exert a strong protective effect. Columns 7 and 8 indicate an apparent radical distribution ratio between IAA and protein of 1 : 20 for the serum albumin and 1 : 11,000 for the leaf protein.

Protective action rather than enhanced inactivation was likewise observed when IAA was irradiated in the presence of plant extracts. Avena coleoptile sheaths and Xanthium leaves and buds were ground, in the cold, for 2 minutes in a mortar with quartz sand. The dispersions were centrifuged at $25{,}000\times g$ for ½ hour at 2° C and the supernatants diluted with water. IAA was added to each diluted extract and the mixtures were transferred to cylinders imbedded in cracked ice. A fifty-fold decrease in IAA sensitivity to x-rays was provided by the presence of ½ strength coleoptile juice (table II, col 4); ⅓ strength Xanthium extract yielded roughly a twenty-fold decrease in initial sensitivity (fig 8). It can be observed from the figure that IAA sensitivity increases as the extract becomes more dilute, and that the curve tends to extrapolate, at high dilutions, to the sensitivity of pure IAA in solution, i.e., initial ionic yields in the region of unity.

DOSE RATE: The tendency for initial ionic yields to fall when initial IAA concentrations were below 0.06 μ moles/ml (fig 5, curve A) was interpreted as reflecting the increased probability of radical recombination and consequent lessening of radical-solute interactions. Extending the time in which equivalent energies were



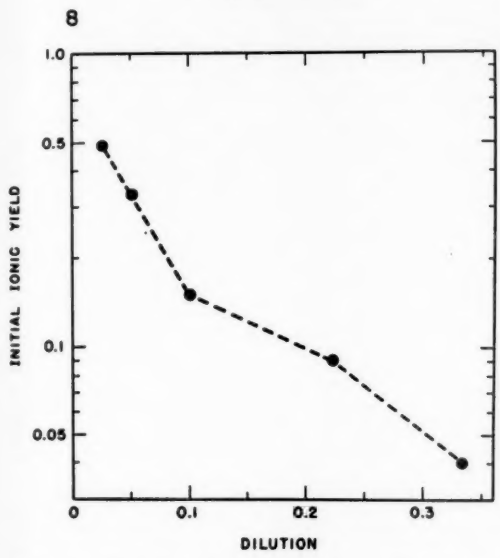
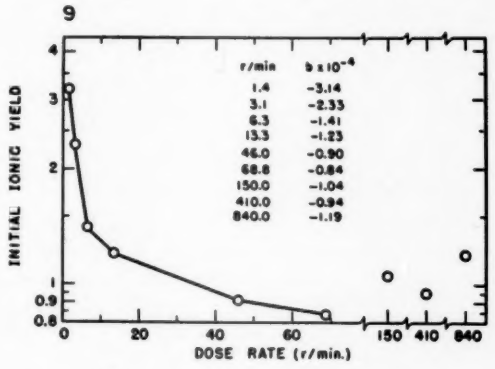


FIG. 7. The effect of recrystallization on IAA inactivation. Initial concentration 53.5 μM. $(M/N)_{r \to 0}$ for cryst. IAA, 0.93; for recryst. IAA, 0.99. The bioassay points represent residual auxin concentrations as determined by the Avena curvature test in aliquots taken from the "cryst" IAA solution at the indicated radiation doses.

FIG. 8. Protective effects of Xanthium extract at various dilutions. Initial ionic yields for IAA inactivation are plotted vs dilution of extract (tissue weight as a fraction of final mixture). Initial IAA concentrations ca 0.1 μmols/ml.

absorbed under continuous irradiation should result in an increased separation of the photon tracks and thereby of the radicals formed. Thus, enhanced radical-solute reaction rates might occur at low solute concentrations when the dose *rate* is lowered. Therefore, IAA solutions in equilibrium with air were irradiated with hard x-rays at dose rates ranging from 1.4 to 840r/min, the intensity range being obtained by varying the target distance and tube current. Sampling was continued until 20 to 30 % inactivation occurred. The effect of dose rate on initial ionic yield, together with the rate constants of IAA disappearance, are given in figure 9. It can be seen that inactivation efficiencies are virtually independent of dose rate above 20r/min. At dose rates below 10r/min the inactivation rate rises. The ionic yields can be extrapolated to a value near 4 at the relatively low rates of energy absorption. The figure also indicates that the dose rates ordinarily used here, as well as in (26), produce ionic yields of about unity.

SENSITIVITY OF AUXIN OBTAINED FROM THE PLANT: The possibility that auxin isolated from the plant might possess a radiosensitivity different from that of synthetic auxin was investigated. The plant auxin was obtained by chromatographic resolution of the ether-soluble acidic fraction of kidney bean plants (see METHODS). Preliminary experiments with pure IAA showed that this auxin could be delimited on a chromatogram either by examination under UV light or by spraying with iron-perchloric acid reagent. By either method the R_f was 0.29 to 0.30. Moreover, 90 to 95 % recoveries of the IAA originally applied could



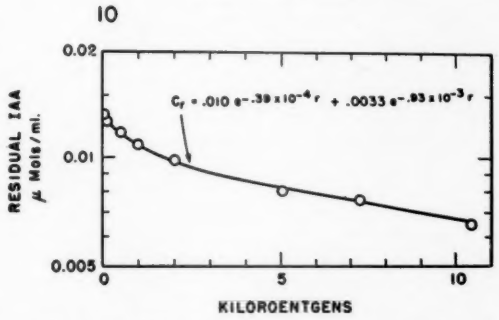


FIG. 9. The effect of x-ray dose rate on initial ionic yield. Initial IAA concentration, 28.6 μM. Tabulated are the rate constants of concentration change at each dose rate.

FIG. 10. Inactivation of auxin obtained from bean plants. The points are mean experimental values, the curve is the calculated fit.

be obtained on elution: the UV outlined spot on a developed strip was cut out and immersed in water, adjusted to pH 3, and both paper and water extracted with ether.

The developed chromatogram of the acid fraction of the plant extract showed several distinct components when surveyed under UV light ($R_f = 0.08$, 0.20, 0.29, 0.61, 0.90, and frontal). However, a parallel guide strip of an aliquot of the plant extract, showing corresponding components under UV, yielded the crimson IAA color only at R_f 0.30 when sprayed with the iron-acid reagent. Segments of a similar strip analyzed by Avena curvature bioassay showed significant activity only at about R_f 0.30. Therefore, this region was cut from the unsprayed chromatogram, eluted as described in the previous paragraph, and taken up finally in 10 ml water. Aliquots of this solution assayed 0.013 μ moles IAA per ml.

The remainder of the solution, in air equilibrium, was irradiated as usual with hard x-rays till approximately 50 % of the original concentration remained. Figure 10 shows the effect of irradiation on this auxin preparation. Here the decrease in concentration as a function of dose departs from the exponential form, unlike the behavior of synthetic IAA (cf., in particular, the lowest curve in figure 3). The concentration-dose relationship for auxin extracted from the plant could be reasonably characterized by two exponentials, and the constants derived for this relation are given in figure 10. Again no unusual auxin radiosensitivity is indicated. Initial inactivation efficiency determined by substitution of the constants in equation (*6*) gives an ionic yield of 1.2.

Discussion

The preceding experiments indicate that the auxin IAA possesses no unusual lability toward ionizing frequencies of the x and γ range. High ionic yields characteristic of an extended chain sequence, in which the inactivation of many solute molecules is initiated by the primary ionization or excitation, were not observed. Inactivation efficiencies were of the same magnitude, irrespective of the method of auxin assay, radiation energy, auxin concentration, oxygen partial pressure, H^+ concentration, or dose rate, in the ranges examined. Recrystallization of the auxin had no effect on its sensitivity. Moreover, the acid auxin extracted from the plant exhibits an initial radiosensitivity in vitro not significantly higher than synthetic IAA. We cannot, therefore, corroborate the high radiosensitivity previously ascribed to IAA (26).

Within the dose ranges examined, the decrease in auxin concentration when pure solutions are irradiated can be accurately characterized as exponential. As is indicated by equations (*11*) and (*14*), this reaction order may be attributed to competition by the products of auxin inactivation as they become an increasing fraction of the total solute. It likewise follows from the above equations that the presence of competing solutes and their inactivation products, which vary in relative reaction affinities with respect to IAA and its products, may yield relationships between C_r and r which cannot be characterized by a single rate constant. This may explain the difference between the reaction order reported here and that indicated by Skoog's data. This interpretation is likewise suggested by our results with the native auxin obtained by chromatography in which the decrease in concentration with dose is non-exponential (fig 10). As a chromatographic eluate, the purity of the auxin used for irradiation in the above experiment may be questioned. Aside from possible introduction of contaminant by manipulative transfers, paper partition chromatography would not have separated components in the plant extract of like mobilities. The above considerations imply that the reaction order of Skoog's data may have resulted from the use of impure preparations of IAA. In addition, they suggest that the apparent exponential decrease in IAA concentration we observed on limited irradiation of auxin in the presence of added solutes might not hold at higher radiation doses.

Slightly increased ionic yields for IAA inactivation were observed with the higher molar ratios of cysteine and glutathione as cosolutes (table II). Cysteine or protein cysteinyl have been suggested as likely reaction sites for auxin function in vivo (12), the carboxyl group and ortho-hydrogen of IAA being postulated as linking with the spatially opposed amino and sulfhydryl groups. Although covalent amide and thiol ester linkages might possibly enhance IAA sensitivity, such bonds would probably not be formed spontaneously in water near neutrality. A carboxyl-amino salt linkage can also be disregarded as a causative factor, since almost identical yields were obtained on irradiating IAA as the pure acid, as the sodium salt, in the presence of ammonium or potassium sulfate, or in the presence of asparagine. It may be suggested that the lack of thiol protective effect and the slight enhancement of IAA inactivation at higher thiol concentrations could result from an accompanying IAA reaction with free thiol radicals, viz., $RSH \rightarrow RS\cdot$; $RS\cdot + IAA \rightarrow RS - IAA + H\cdot$. In this connection, an enhancement of a methyl linoleate oxidation by x-radiation in the presence of glutathione has been reported (22).

The observations on the protective effects of added substances are pertinent to the radiosensitivity of IAA in vivo. Though the auxin has no unusual sensitivity when irradiated as a pure solute, table II indicates that its pZ, or affinity for activated water, is high among those low molecular weight cosolutes examined. In broad terms, it appears more readily oxidized by x-radiation. On the basis of the net auxin inactivation, the generalization would apply even to glutathione and cysteine, whose lability (17, 23) and protective action (22, 24) have been demonstrated elsewhere. Yet, a number of the solutes do protect the auxin when in suitable molar ratios. In this respect, the major protein component of leaf cytoplasm (28), Fraction I protein, with its relative pZ of ca 10^4, is highly protective. Similarly, in Dale's study of the protective action of various cosolutes (2), both the

apoenzyme and prosthetic group of D-amino acid oxidase had the highest radical affinities of the many compounds examined. If we consider that free auxin occurs in leaf tissues at concentrations on the order of 10^{-8} gm moles per kg, surrounded by a heterogeneous mixture of potentially reactive solutes, the proteins alone being in over 10^4 mole excess, it appears improbable that the absorption of low doses of ionizing radiation would result in significant auxin depletion by direct molecular inactivation. Indirectly substantiating this conclusion are the results obtained by irradiating IAA in the presence of coleoptile (table II) and leaf (fig 8) extracts. The diluted aqueous extracts, containing only a small fraction of the plant's soluble components, showed marked protective action. We therefore believe that an explanation other than direct auxin photolysis probably accounts for the lowering of auxin levels (26) in plants exposed to low doses of ionizing radiation.

Emphasis has been placed recently on the ratio of solute yields (R) obtained by irradiating dilute solutions in the presence and absence of oxygen. Since identical dosimetry was used in this study to evaluate the effects of oxygen saturation and air evacuation on IAA yields, the ratios of yields were calculated. Table III lists the R values obtained at four initial IAA concentrations. A ratio between 3 and 4 is not inconsistent with mechanisms proposed (6, 17) to account for solute oxidations by ionizing radiation. Hydroxyl radicals are formed in the absence of dissolved O_2 when water is irradiated by γ- or hard x-rays. In the presence of O_2, the solute could be oxidized not only by OH radicals but also by concomitantly formed hydroperoxyl radicals and ions. This would yield a theoretical R value of 4 (Amphlett in (6)). A lower R value may be attributed to the back reactions plus pairwise combination of like radicals in zones of high free radical concentration (6, 14). The ionic yields obtained by irradiating aerated solutions of IAA at low dose rates (fig 9) approach the above theoretical value. Figure 9 likewise suggests that, at an initial IAA concentration of 29 μM, the radical recombination rates attain and maintain a steady state for a wide range of dose rates above ca 20r/min.

Although IAA apparently disappears in the presence of H_2O_2 or organic peroxides, Siegel and Weintraub (25) have shown that no actual destruction of the auxin takes place. The "inactivation" derives from the intereference by the peroxides with both biological and colorimetric assays for IAA. Their observations raised the possibility that the peroxide produced in irradiated water would cause spurious values for IAA destruction through anomalous assay values. However, it will be shown elsewhere that a) H_2O_2 is probably not involved in auxin destruction by ionizing radiation, b) the peroxide levels found under our irradiation conditions are well below the concentrations where assays are affected, and c) structural alteration of the IAA molecule does occur.

TABLE III

RATIO OF INITIAL IONIC YIELDS BETWEEN OXYGEN SATURATED AND EVACUATED SOLUTIONS OF IAA

INITIAL CONC	$(M/N)_{r\to 0}$		R
	$+O_2$	$-O_2$	
μmols/ml			
0.0355	1.29	0.42	3.1
0.089	1.33	0.37	3.6
0.832	1.16	0.32	3.6
4.29	1.10	0.29	3.8

SUMMARY

The in vitro sensitivity of indoleacetic acid (IAA) to x- and γ-radiation has been re-examined by a kinetic approach. No unusual lability of the auxin was observed on varying the auxin concentration or purity, oxygen concentration, H^+ concentration, radiation energy, dose rate, or method of assay. Ionic yields were near unity. Acid auxin extracted from the plant exhibits an intrinsic radiosensitivity not significantly higher than synthetic IAA. The effects of several cosolutes and plant extracts on IAA destruction were examined to determine the relative radical affinity of the auxin. The above observations indicate that an explanation other than direct auxin photolysis probably accounts for the lowering of auxin levels in plants exposed to low doses of ionizing radiation.

An approximation is described for determining initial ionic yields where changes in solute concentration are nonlinear with respect to radiation dose.

We are particularly indebted to Sylvanus A. Tyler of this laboratory for his aid in the mathematical derivations. We also wish to thank Leonidas D. Marinelli and Edwin J. Hart for their aid and suggestions, Joan M. Gurian for assistance in statistical analyses, and Emil G. Johnson for technical help in the x-irradiations.

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THE CORRELATION OF DIFFERENT ASPECTS OF AUXIN ACTION[1]

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An auxin has been defined by Thimann (10) as "an organic substance which promotes growth (i.e., irreversible increase in volume) along the longitudinal axis, when applied in low concentrations to shoots of plants freed as far as practical from their own inherent growth-promoting substance. Auxins may, and generally do, have other properties, but this one is critical."[2] This definition emphasizes the aspect of auxin activity which was first studied by plant physiologists and which becomes particularly obvious when a stem or petiole responds with curvature to unilateral illumination. It was this curvature that first aroused the interest of Charles Darwin (2) and led to his classical studies on the power of movement in plants. This same curvature led Boysen-Jensen (1), thirty years later to his experiments with decapitated oat coleoptiles in which he demonstrated the capacity of the curvature stimulating influence to diffuse through a gelatin barrier and finally to the experiments of Went (12) who proved that agar on which the coleoptile tips had been placed contained a substance which caused curvature in decapitated coleoptiles.

It is therefore logical from a purely historical point of view to emphasize that aspect of auxin action which manifests as elongation growth. But, as Thimann points out in the same paper, the effects of auxin on plant cells are numerous and are by no means confined to stimulating growth in length. Stimulation of cell division in the cambium, initiation of adventitious roots, inhibition of growth of axillary buds, stimulation of parthenocarpic development in fruits are all aspects of the action of auxin. It is this multiplicity of effect which makes the action of an auxin so difficult to define and its mechanism so hard to explain. Nor is the problem made any simpler by the fact that there are many unrelated chemical compounds which function as auxins.

In the present work the technique of cultivating sterile segments of sunflower hypocotyl on nutrient agar was utilized (3). Because the segments were sterile their behavior could be studied over a period of several weeks which made possible the observation of other more slowly induced auxin effects besides the rapidly manifested effect of stem elongation. These

[1] Received October 9, 1954.

[2] More recent definition of auxin was given in Plant Physiol. 29: 307, 1954. This does not differ in any essential feature from the earlier definition given by Thimann.

effects were cellular proliferation and production of adventitious roots. The work was planned to demonstrate how these three aspects of auxin activity are affected: 1) by the position, apical or basal, of the fragment in the hypocotyl, 2) by the end, apical or basal, to which the auxin was applied, 3) by the concentration of auxin, 4) by the presence or absence of light, 5) by the age of the hypocotyl from which the fragments were taken.

Materials and Methods

Sunflower seeds (var. Russian giant) were sterilized with alcohol followed by Clorox. The embryos were removed aseptically and placed on 10 ml of 1 % agar containing mineral salts in the proportions used by Hildebrandt, Riker and Duggar (6) in 22 × 175 mm tubes. Embryos to be used for experiments in light were placed in the light given by four 40-watt white fluorescent lamps at a distance of 2 feet from the light source, those to be used for experiments in darkness were germinated in complete darkness. Embryos were used when 3 or 5 days old. The hypocotyl was removed aseptically, severed just below the cotyledons and immediately above the roots. The upper and lower 5 mm of the hypocotyl was then cut off. These hypocotyl segments, to be referred to as the apical and basal fragments respectively, were then placed on 1 % agar with mineral salts, as above, containing also 2 % sucrose and dispensed in 10 ml amounts in 18 × 150 mm tubes. The agar was supplemented with indole-3-acetic acid (IAA) at concentrations of 50, 10, 1, 0.1 and 0.01 μgm/ml. In the series cultured in darkness the preparation of the hypocotyl fragments was carried out by the light of a 40-watt red Safelite, and the tubes were then incubated in darkness at 24° C. The series in light was incubated at a distance of 2 feet from a group of 4 fluorescent lamps.

Growth in length was measured after 48 hours and after 7 days. Measurements were made with a microscope equipped with a moveable stage and an ocular micrometer. Determinations of fresh and dry weights and of numbers of adventitious roots were made after 7 or 14 days.

Results

In figure 1 the response of the sunflower hypocotyl fragments to IAA has been summarized. Elongation occurred only in the apical fragments and was a rapid process, completed within the first 48 hours. Basal fragments did not elongate at all. Maximum elongation was obtained when the apical fragment was placed with the apical end on the agar. When the basal end was placed on the agar very little elongation occurred. This was no doubt the result of polarity in auxin transport. Continuous light reduced elongation but did not eliminate it. The peak of the response occurred at a concentration of from 0.1 to 0.01 μgm/ml IAA. It was strongly inhibited by concentrations of 1 μgm/ml or over. Fragments from 3-day-old hypocotyls had a slightly greater capacity to elongate than had those from 5-day-old hypocotyls.

Table I

Growth in 14 Days of Sunflower Hypocotyl Fragments Cultivated in Darkness on Agar Containing IAA (Means of 10 Values)

End in contact with agar	Measurements	IAA conc, μgm/ml 50	10	1	0.1	0.01	0
	Apical fragment						
Apical	Fresh wt, mg	86	247	195	109	77	55
	Length, mm	9.2	9.5	10.8	15.2	14.4	10.4
	Roots/fragment	0	0	0	0	0	0
Basal	Fresh wt, mg	122	379	227	202	114	43
	Length, mm	9.2	10.6	10.4	10.0	10.4	8.0
	Roots/fragment	0	0	0	1.3	0	0
	Basal fragment						
Apical	Fresh wt, mg	41	80	101	117	92	52
	Length, mm	5.4	6.0	6.4	6.4	6.4	6.0
	Roots/fragment	0	0	0.3	2.3	2.3	0.2
Basal	Fresh wt, mg	48	92	123	113	104	59
	Length, mm	5.0	5.0	6.4	6.4	6.0	5.8
	Roots/fragment	0	0	0	0.9	1.8	0.3

Fresh and dry weights of the fragments were influenced by IAA in a different way from elongation. Apical fragments, in general, showed a peak response at from 1 to 10 μgm/ml IAA whereas the peak for basal fragments was at 0.1 to 1 μgm/ml. Response in both cases was greater when the basal end of the fragments rested on the agar. Response to the lower concentrations of auxin was reduced by light.

Root initiation was greater in basal than in apical fragments and reached a maximum value when from 0.01 to 0.1 μgm IAA was applied to the apical end of the basal fragments (table I). Application of auxin to the apical end of such fragments resulted in the emergence of roots from both top and bottom of the fragment and values as high as ten roots per fragment were sometimes recorded. When auxin was applied to the basal end of the fragment only one set of roots was produced.

The appearance of fragments grown on various concentrations of IAA is shown in figure 2. These fragments were cultured in darkness for 14 days on agar containing 50, 10, 1, 0.1, 0.01 μgm/ml IAA. The quantitative aspects of their response are shown in table I. The highest IAA concentration (50 μgm/ml) was toxic, the fragments becoming converted to waterlogged masses of tissue incapable of further growth; 10 μgm/ml was toxic for the basal fragments but growth-promoting for the apical fragments; 1 μgm/ml was growth-promoting for both apical and basal fragments but inhibited elongation of the apical fragments. The concentration of 0.1 μgm/ml stimulated elongation of the apical fragment when applied to the apical end. When applied to the basal end it

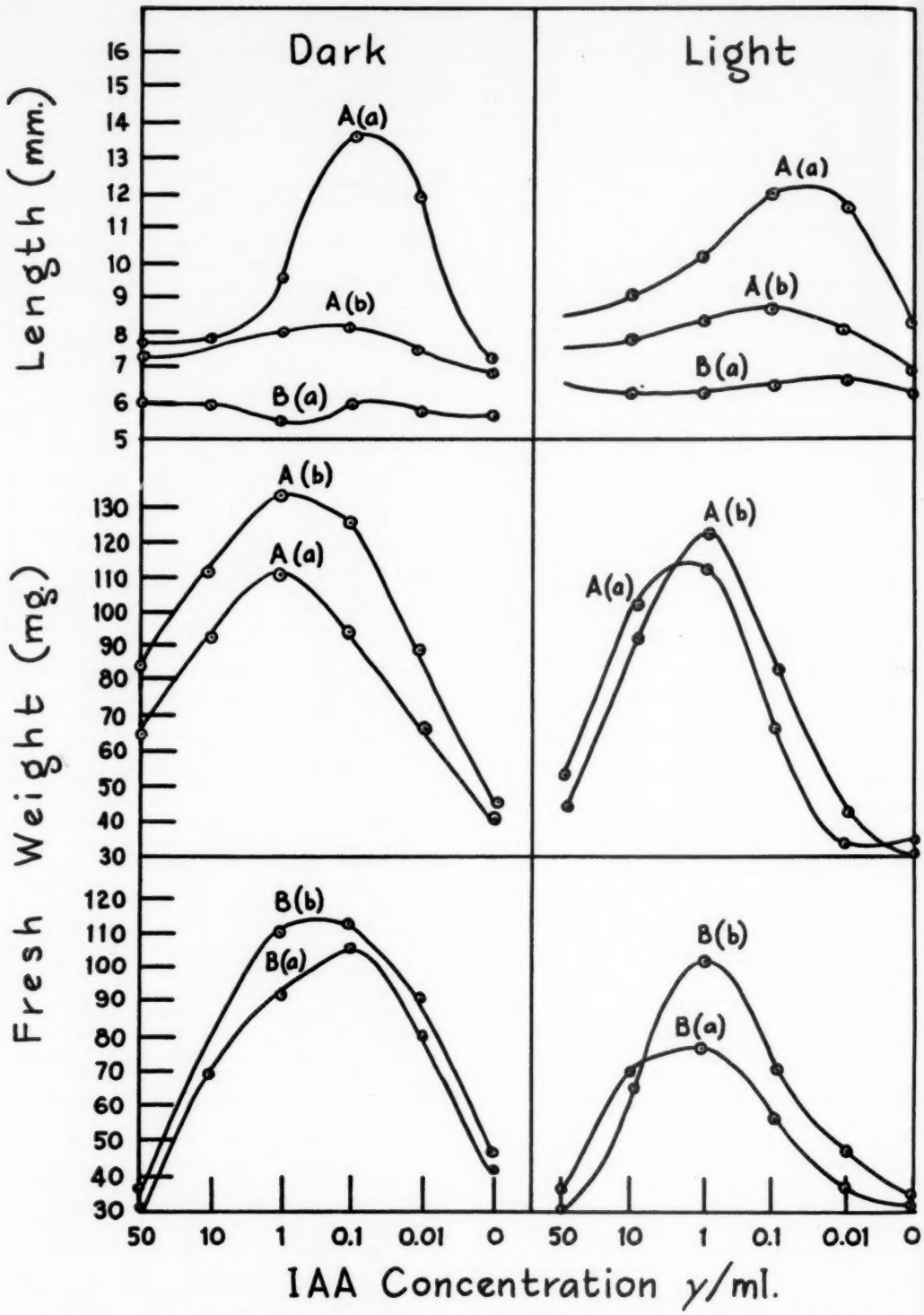

FIG. 1. Increase in length and in fresh wt in 7 days of sunflower hypocotyl fragments cultured on IAA. A—apical fragment, B—basal fragment, (a)—apical end on agar, (b)—basal end on agar.

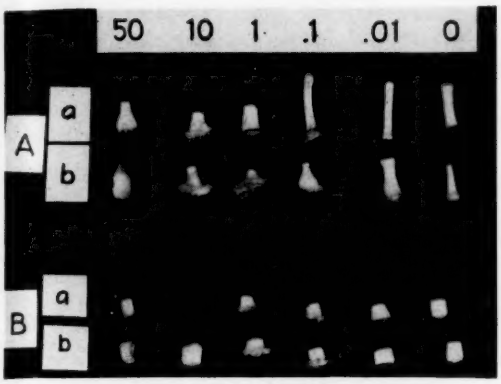

FIG. 2. Appearance of sunflower hypocotyl fragments after 14 days culture on IAA in darkness. Concentration of IAA in μgm/ml. A—apical fragment, B—basal fragment, (a)—apical end on agar, (b)—basal end on agar.

stimulated intense cellular proliferation which caused the basal end of the apical fragment to swell to several times its original diameter though very little elongation occurred. In the basal fragments this concentration of IAA stimulated the greatest root initiation. Even 0.01 μgm/ml was capable of stimulating elongation in the apical fragments when applied to the apical end and root initiation when applied to the basal end.

DISCUSSION

All the aspects of auxin activity which have been studied here have been well known for years and were described in full by Went and Thimann (13) in their classical monograph on phytohormones. The problem is essentially one of explaining why a single chemical substance, indole acetic acid, evokes more than one type of response from the tissue on which it acts. Under one set of conditions growth in length occurs, under another set of conditions cambial proliferation predominates and under a third production of adventitious roots is the outstanding characteristic of the reaction. Undoubtedly auxin concentration is of great importance in determining the kind of response which occurs. Gautheret (4) emphasized this point in 1945 and drew up a general scheme of auxin activity defining a concentration from 10 to 1000 μgm/ml as producing isodiametric growth of cells and bud inhibition, from 0.1 to 10 μgm/ml as rhizogenic, from 0.001 to 0.1 μgm/ml as inducing cellular multiplication and from 0.0001 to 0.01 μgm as promoting cellular elongation. The behavior of the sunflower hypocotyl fragment fits fairly well into this scheme. Isodiametric cell enlargement was observed close to the toxic concentration of IAA at about 10 μgm/ml, root initiation reached its peak between 0.1 and 0.01 μgm/ml, elongation was greatest at from 0.1 to 0.01 μgm/ml. The growth-promoting effect of IAA, using this term to refer only to weight increase, reached its peak between 1 and 10 μgm/ml.

Concentration of auxin is only one of the factors which influence the mode of response of plant tissue. That different regions of a growing stem elongate at different rates has been known to botanists ever since the studies of Sachs (8). The difference is not due to the fact that tissue near the apex receives more auxin than tissue near the base. As the present experiments show even the highest concentrations of IAA will not cause the elongation of fragments taken from the basal end of the stem. An explanation of the gradient of response to auxin might be sought in the findings of Galston and Dahlberg (5) who have shown that IAA oxidase, which converts IAA to a physiologically inactive product (9), increases in concentration in the tissues of the etiolated pea epicotyl as one proceeds from the apex towards the base. The basal fragments of the sunflower hypocotyl, however, have not lost their capacity to respond to IAA. What they have lost is their capacity to elongate. They respond to auxin only by increase in weight and by root initiation.

So far as root initiation by auxin is concerned, it is hard to avoid the conclusion put forward by many students of auxin activity, especially by Went (11), that auxin-induced rooting results from the interaction of auxin and some other factor which tends to accumulate in the basal portions of the stem and to be almost totally lacking in the apical regions. Previous work on the growth capacity of the sunflower hypocotyl (3) suggests that this substance needed for auxin induced root initiation reaches its maximum concentration between 30 and 60 mm from the apex of a 100 mm hypocotyl and is somewhat reduced in the lowest 5 mm of the stem. Although Went's concept of rhizocaline may require some modification, it does appear that some such factor must interact with auxin before adventitious roots can be initiated and that this co-factor tends to accumulate in the lower portion of the stem and to be absent or present in reduced amounts in the upper portion.

As regards theories of the mechanism of auxin action such as the two-point attachment theory (7), it would seem necessary to take into account the fact that different aspects of auxin activity show peak responses at widely separated concentration levels. Thus the peak for elongation occurs, for sunflower hypocotyl tissue, at between 0.1 and 0.01 μgm/ml, but the peak for weight increase occurs between 10 and 1 μgm/ml for apical fragments and 1 and 0.1 μgm/ml for basal fragments. The action of IAA does not merely become inhibitory as the concentration is raised. It inhibits one form of growth (elongation) but enhances another type of growth (weight increase due to cellular proliferation). Any satisfactory theory of auxin action must take this fact into account.

SUMMARY

1. Three aspects of auxin activity, namely, stem elongation, increase in fresh and dry weight and initiation of adventitious roots, have been studied.

2. Stem elongation took place only when the auxin was applied to the apical end of the apical 5 mm of the hypocotyl. Maximum elongation occurred at a concentration of from 0.1 to 0.01 μgm/ml IAA. Higher concentrations inhibited elongation. Fragments from the base of the hypocotyl failed to elongate on any concentration of auxin. Elongation was reduced but not eliminated by light.

3. Maximum increase in fresh weight of the fragments took place at between 1 and 10 μgm/ml IAA in the apical fragments, and this increase was greater when the IAA was applied at the basal rather than the apical end of the fragment. Fragments from the base of the hypocotyl responded with maximum weight increase to a concentration of 0.1 to 1.0 μgm/ml IAA.

4. Initiation of roots was greatest at a concentration of 0.1 μgm/ml IAA and attained a maximum value when the IAA was applied to the apical end of the basal hypocotyl fragments.

5. It is concluded that the strictly growth-promoting activity of IAA, as measured by weight increase reaches a peak at between 1 and 10 μgm/ml. Its auxin activity reaches a peak between 0.01 and 0.1 μgm/ml. The existence of two such widely separated peaks of activity must be taken into account in any comprehensive theory of auxin action.

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CALCIUM–MAGNESIUM NUTRITION WITH SPECIAL REFERENCE TO SERPENTINE SOILS[1]

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Much evidence has been presented to show that the growth of plants is markedly affected by the proportions of the exchangeable cations which are present in the soil. This evidence has been reviewed recently in detail by Mehlich and Coleman (9) and Stout and Overstreet (13). The effects on plant growth of the degree of Ca saturation of the soil colloids has been emphasized in such studies. They show that Ca absorption is decreased and plant growth limited when Ca saturation is at low levels, regardless of the complementary ions which are present on the colloids. There are definite differences, however, between the influences of adsorbed H, Mg, K, or Na on Ca availability in the soil. The present study is concerned only with the cases in which Ca and Mg are the principal exchangeable cations, and in which a raising or lowering of either of these is reflected in a complementary increase or decrease in the other. This emphasis on Ca–Mg relationships was prompted by the interest of the authors in soils derived from serpentine or related rocks, which are very high in Mg.

A number of studies have been concerned specifically with Ca–Mg interrelationships in the soil. Hunter (6) observed no effect on the yield of alfalfa when he varied the Ca/Mg ratio between 1/4 and 32/1, although Ca and Mg uptakes were altered by the changes in the soil cations. Using Ca–Mg soils, Vlamis (15) demonstrated that yields of lettuce and barley were dependent upon Ca saturation up to a level of about 20 %, above which no further response was evidenced. In experiments on several soils using Ladino clover, Giddens and Toth (2) found that reduction of the Ca saturation from 65 to 25 % restricted yields somewhat and much reduced the absorption of Ca.

It has been recognized for many years that crop plants grow poorly on serpentine soils. The early work of Loew and May (8) and the later investiga-

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tions of Vlamis and Jenny (16) and Vlamis (15) indicated that the principal reason for the infertility of these soils is their low Ca/Mg ratio or low Ca saturation. Apparently crop plants are unable to absorb sufficient Ca from these soils for proper growth. Various factors affecting plant growth on serpentine soils and the dominant role of Ca availability in these habitats were discussed recently by the senior author (18).

Although agricultural plants grow poorly on serpentine soils, a considerable number of native species thrive in this medium. Obviously the latter must differ physiologically from crop plants, and perhaps some phase of their Ca–Mg nutrition is the most likely point of difference. In order to investigate this possibility, the Ca–Mg nutrition of several native serpentine species is compared with that of some common agricultural plants in this paper.

Materials and Methods

Two natural soils were used for the pot culture experiments. They are characterized as follows:

No. 30. A shallow, primary, reddish-brown soil overlying serpentine rock in Lake County, California, classified in the Henecke soil series. The pH is 6.8 as read on the saturation paste with a glass electrode; the cation exchange capacity is 16.1 meq/100 gm; Ca saturation is 14 %; Ca/Mg ratio in the cation exchange complex is 0.16. An x-ray diffraction pattern indicated that the 2 μ colloidal fraction is predominantly kaolinitic.

No. 85. A shallow, primary, gray soil overlying serpentine rock in Chelan County, Washington. The pH is 6.4; the cation exchange capacity 24.4 meq/100 gm; Ca saturation is 32 %; and the Ca/Mg ratio in the cation exchange complex is 0.48.

After screening through ¼-inch mesh to remove rock, 15 kgm lots of the above soils were reconstituted with respect to exchangeable cations by leaching in inverted bell jars with appropriate mixtures of $CaCl_2$ and $MgCl_2$ (17). In most cases 36 liters of leaching solution with a total salt concentration of 0.25 molar were used. For the very low Ca soils, the solution contained $MgCl_2$ alone. After reconstitution with the chloride mixture, the soil lots were leached with water until free of chloride to the $AgNO_3$ test, then dried, screened again, and mixed. Samples were analyzed for exchangeable cations by ammonium acetate extraction and determination of the bases in the extract. Although no K was included in the leaching solutions, some residual K persisted in the soil after treatment. The cation status of the soil lots after reconstitution is given in table I. A slightly different but similarly prepared series was used for the experiment with tomato on Soil 30. The range of Ca saturation extends from below that of any naturally occurring soil up to over 80 %, which is higher than that present in most fertile agricultural soils.

The pot culture methods used followed those of Jenny et al (7). Six-inch clay pots, coated on the inside with black asphaltum varnish and on the outside with the asphaltum and a coat of aluminum enamel, were used as containers, each filled with 1600 gm of soil. Before the soil was placed in the pots, the following amounts of fertilizer salts, in dilute water solution, were thoroughly incorporated into each pot of soil by hand mixing: Soil 30—0.700 gm NH_4NO_3, 0.584 gm $Ca(H_2PO_4)_2 \cdot H_2O$ and 0.304 gm K_2SO_4; Soil 85—0.500 gm NH_4NO_3, 0.520 gm $NH_4H_2PO_4$, 0.295 gm K_2SO_4. Irrigation was with distilled water as needed. Solution cultures were conducted in 2-qt mason jars, using the technique of Hoagland and Arnon (5).

Table I

Cation Exchange Data (2-mm Fraction)

Soil lot no.	Cations				Exchangeable Ca **
	Ca	Mg	K	Sum	
	meq/100 gm dry wt				%
30-A	0.98	15.0	0.073	16.1	6.1
30-B	1.18	14.6	0.074	15.9	7.3
30-C	1.76	14.3	0.064	16.1	10.9
30-D *	2.18	13.8	0.078	16.0	13.5
30-E	2.97	13.0	0.072	16.0	18.4
30-F	4.08	11.8	0.073	16.0	25
30-G	7.99	8.3	0.045	16.3	50
30-H	13.2	3.1	0.071	16.4	82
85-I	0.69	22.9	0.20	23.8	2.8
85-J	0.90	22.9	0.20	24.0	3.7
85-K	1.22	22.9	0.21	24.3	5.0
85-L	1.73	22.0	0.20	23.9	7.1
85-M	2.36	20.4	0.20	23.0	9.7
85-N	5.05	17.8	0.20	23.1	20.7
85-O	5.16	18.2	0.20	23.6	21.1
85-P	7.12	15.8	0.20	23.1	29.2
85-Q *	7.85	16.5	0.20	24.6	32.2
85-R	7.96	16.6	0.20	24.8	32.6

* This lot is the natural field soil, leached only with water. Other lots were reconstituted by leaching with salt solutions.

** Percent computed on the basis of NH_4 adsorption capacities: Soil 30, 16.1 meq/100 gm; Soil 85, 24.4 meq /100 gm.

The plants used were common sunflower (*Helianthus annuus* L. var. Russian Mammoth), tomato (*Lycopersicum esculentum* Mill. var. Marglobe), common buckwheat (*Fagopyrum esculentum* Moench.), and three annual species native to the California coast range serpentine areas. The latter were *Helianthus bolanderi* A. Gray (a serpentine race, population No. 643 of Heiser (3)), *Streptanthus glandulosus* Hook., and *S. g.* var. *pulchellus* (Greene) Jepson. The seeds were germinated in silica sand, and the seedlings irrigated with tap water supplemented by frequent applications of a complete nutrient solution. When large enough to transplant, seedlings selected for uniformity were transferred to the soils or solutions. One seedling per pot or jar was used for sunflower or tomato, two for the native species except 3 for *H. bolanderi* on Soil 85, and 5 for buckwheat.

Treatments were run in quadruplicate in solution experiments, and in triplicate in soil experiments.

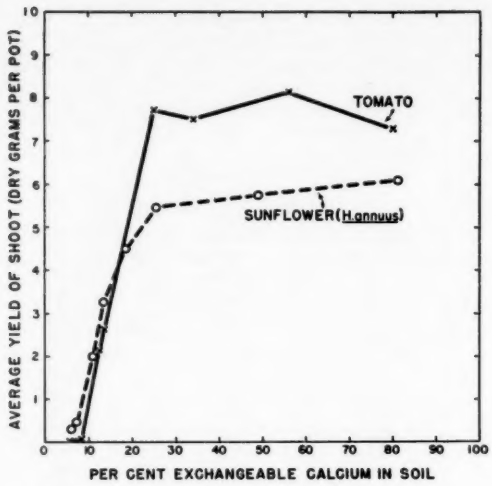

FIG. 1. Yields of tomato and sunflower as affected by % exchangeable Ca in Soil 30.

The plants were grown on greenhouse benches in random blocks during the spring and summer months. Harvest was made after 5 to 10 weeks, depending on the species, but in all cases at about the time floral buds were being initiated. The shoots were harvested, separated into two portions on the basis of 1) good leaves and 2) stems and senescent leaves, dried at 70° C, ground to pass through a 40-mesh screen, and analyzed for various constituents.

In all analyses, Ca was determined by titration of the Ca oxalate precipitate with permanganate, and K was precipitated as the cobaltinitrite and titrated with permanganate (4). Magnesium was determined in soils by iodimetric titration of the $MgNH_4AsO_4$ precipitate (14), and in plant tissue by a modified molybdivanadate colorimetric estimation of the PO_4 in the $MgNH_4PO_4$ (10). The modification was suggested by C. M. Johnson and E. Epstein, Dept. of Plant Nutrition, University of California, Berkeley. Ten ml of the following combined reagent is used in 100 ml of the reactant mixture: dissolve 46 ml H_2SO_4, 10 ml HNO_3, 0.83 gm NH_4VO_3, and 17 gm ammonium molybdate in 500 ml of water, then make to 1000 ml. Cation exchange procedures used were those recommended by Peech et al (12) with minor modifications. Plant tissue was digested by a mixture of nitric and perchloric acids in preparation for analyses.

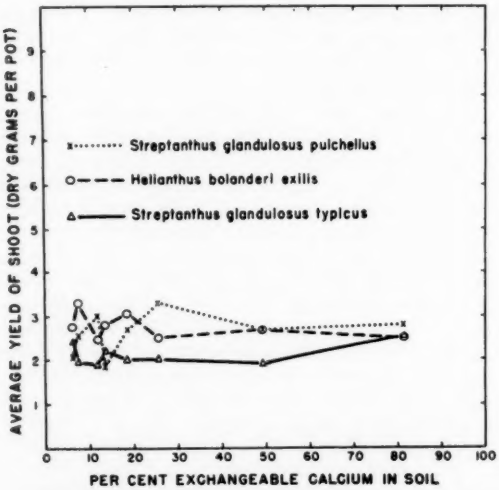
AVERAGE YIELD OF SHOOT (DRY GRAMS PER POT)
Streptanthus glandulosus pulchellus
Helianthus bolanderi exilis
Streptanthus glandulosus typicus
PER CENT EXCHANGEABLE CALCIUM IN SOIL

FIG. 2. Yields of native serpentine species as affected by % exchangeable Ca in Soil 30.

RESULTS

POT CULTURE EXPERIMENTS USING SOIL 30: Tomato, sunflower, and the 3 serpentine species were grown in greenhouse pot cultures on the lots of Soil 30 (table I). The effect of the changes in Ca saturation on yields of these species is shown in figures 1 and 2. Representative data giving Ca, Mg, and K contents of the leaf tissue are given in table II.

The yields of the crop plants decreased markedly at exchangeable Ca levels below 20 %, and below 10 % there was almost no growth (fig 1). In all cultures of these species at Ca levels of 10 % or below, there were acute symptoms of Ca deficiency, such as curling and involution of the leaf margins and failure of leaf primordia to expand. Even at Ca levels up to 12 to 15 %, incipient deficiency symptoms, such as wrinkling of some leaves, were evident.

TABLE II

YIELD AND CATION COMPOSITION AS AFFECTED BY % EXCHANGEABLE CA IN SOIL 30

% Ca in soil cations	Average yield/pot ** (gm dry wt)	Cations in leaf tissue (meq/100 gm dry wt)			
		Ca	Mg	K	Sum
Common sunflower (Helianthus annuus)					
6.1	0.31	21.9	269	37	328
7.3	0.47	18.2	254	55	327
10.9	2.00	17.1	123	97	237
13.5 *	3.26	23.5	123	97	244
18.4	4.51	38.4	139	69	246
25	5.46	58.2	116	72	246
50	5.75	98.3	83	73	254
82	6.09	137	68	81	286
Native sunflower (H. bolanderi)					
6.1	2.75	31.9	100	99	231
7.3	3.30	39.5	97	103	240
10.9	2.44	53.5	85	109	248
13.5 *	2.80	64.8	100	107	272
18.4	3.07	78.1	68	108	254
25	2.50	95.1	68	100	263
50	2.69	95.5	50	110	256
82	2.50	113	31	108	252

* Natural field soil.

** *H. annuus*, 1 plant per pot; *H. bolanderi*, 2 plants per pot.

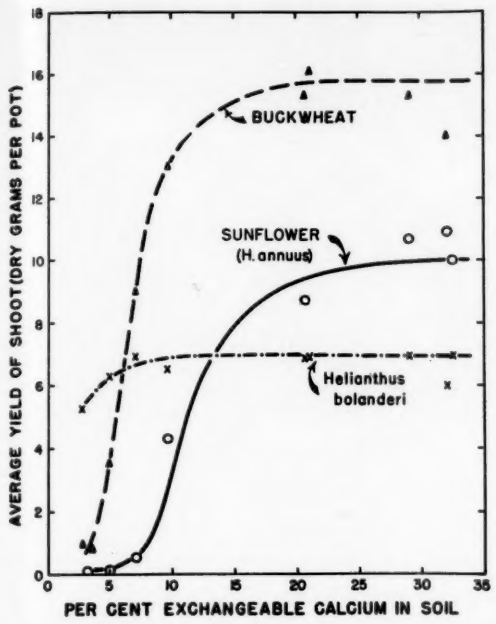

FIG. 3. Yields of *Helianthus annuus, H. bolanderi,* and buckwheat as affected by % exchangeable Ca in Soil 85.

In the serpentine species, the response to the varying Ca levels was quite different from that shown by the crop plants. Although the yield data are somewhat erratic, because of the difficulty of obtaining uniform growth in the native plant seedlings, there is no indication of depression of yield even at the lowest Ca level used, 6 % (fig 2). In some of the Streptanthus plants, however, even though yield was not significantly affected, there were signs of incipient Ca deficiency at this lowest Ca level.

An examination of the leaf composition of the plants from these experiments (table II) shows that at comparable Ca levels the cultivated sunflower (*Helianthus annuus*) absorbed less Ca and more Mg than the serpentine native sunflower (*H. bolanderi*). Analyses of leaf tissue of the other species studied showed similar relationships. Tomato and sunflower thus in every case had lower percentages of Ca and higher percentages of Mg in the leaf tissue than the native species on all of the soil lots except those of 50 and 82 % Ca.

POT CULTURE EXPERIMENTS USING SOIL 85: The results of experiments using Soil 30 indicated that the growth of serpentine species should be studied at levels of Ca even lower than 6 % saturation. Thus a number of lots of Soil 85 were prepared that ranged down to 2.8 % Ca saturation (table I). This was as low as Ca could be reduced by the leaching method employed. On this soil sunflower, buckwheat, and the serpentine species, *Helianthus bolanderi,* were grown. Buckwheat was included because of its known tolerance of infertile soils. The yield responses of these species to the varying Ca saturation are shown graphically in figure 3. These graphs indicate that the nature of the responses of sunflower and buckwheat to the level of exchangeable Ca are similar, but Ca would apparently have to be reduced further to depress the yield of *H. bolanderi* appreciably. The plants obviously form a series in their tolerance of low Ca, with *H. bolanderi* the most tolerant, buckwheat intermediate, and common sunflower the least tolerant. Common sunflower showed incipient Ca deficiency on the 21 % Ca soil and severe deficiency on 9.7 % Ca and below. Buckwheat showed incipient Ca deficiency at 9.7 %, and severe deficiency at 5.0 % Ca. *H. bolanderi* showed mild Ca deficiency at the 2.8 % and 5.0 % Ca levels. The appearance of the common and native sunflower plants is illustrated in figure 4.

The yields and composition of the leaf tissue of the plants grown in the experiments with Soil 85

TABLE III

YIELD AND CATION COMPOSITION AS AFFECTED BY % EXCHANGEABLE CA IN SOIL 85

% Ca in soil cations	Average yield/pot †† (gm dry wt)	Cations in leaf tissue (meq/100 gm dry wt)			
		Ca	Mg	K	Sum
Common sunflower (Helianthus annuus)					
3.3 *	0.10 **	...	...	..	...
5.0	0.16 **	...	...	..	...
7.1	0.54	19.0	285	66	370
9.7	4.27	17.8	153	69	240
20.7	8.68	25.9	121	51	198
29.2	10.7	37.0	105	60	202
32.2 †	10.9	44.7	121	59	225
32.6	10.0	39.7	137	57	234
Common buckwheat (Fagopyrum esculentum)					
2.8	0.95	11.3	303	16	330
3.7	0.81	13.9	387	15	416
5.0	3.50	14.3	339	25	378
7.1	8.96	20.4	333	22	375
9.7	13.0	23.5	253	25	302
20.7	15.3	57.9	229	19	306
21.1	16.1	53.9	223	26	303
29.2	15.3	75.1	189	27	291
32.2 †	14.0	89.2	222	24	335
Native sunflower (Helianthus bolanderi)					
2.8	5.29	15	177	65	257
5.0	6.33	25	212	55	292
7.1	6.92	40	313	50	403
9.7	6.55	63	346	56	465
20.7	6.86	101	205	54	360
21.1	6.91	88	160	71	319
29.2	6.95	77	82	75	234
32.2 †	6.00	136	148	81	365
32.6	6.95	138	181	68	387

* This lot was a mixture of equal weights of lots 85–I and 85–J (cf table I).

** Insufficient material for leaf analysis.

† Natural field soil.

†† Number of plants per pot: *H. annuus,* 3; buckwheat, 5; *H. bolanderi,* 3.

FIG. 4 (*above*). Common sunflower (*Helianthus annuus*) grown in Soil 85. Ca saturation from left to right: 32.2% (field soil), 7.1, 9.7, 20.7, 29.2, and 32.6%. Curling and buckling of leaves from Ca deficiency is apparent at the lower Ca levels. Plants grown at 3.3 and 5.0% died before the picture was taken. (*Below*). Native sunflower (*H. bolanderi*) grown on Soil 85. Ca saturation from left to right: 32.2% (field soil), 2.8, 5.0, 7.1, 9.7, 21.1, 29.2, 32.6, and 20.7%. Growth was good even at the lowest Ca levels.

are given in table III. At comparable Ca levels, *Helianthus bolanderi* absorbed appreciably more Ca than the other species, but in contrast with results using Soil 30, absorbed somewhat more Mg than *H. annuus* at comparable levels of Ca in the soil. Buckwheat generally absorbed the most Mg of the three species grown on Soil 85, but showed an absorption of Ca which lay between the uptakes by the two sunflower species.

SOLUTION CULTURE EXPERIMENTS: In order to compare the absorption of Ca and Mg from culture solutions with that from soils, common sunflower and buckwheat were grown on a series of culture solutions, the compositions of which are given in table IV.

Table IV

Compositions of Culture Solutions (Moles/Liter)

Constituents	Solution			
	A	B	C	D
$NH_4H_2PO_4$	.001	.001	.001	.001
KNO_3	.006	.006	.006	.006
$Ca(NO_3)_2$	.004	.0005	.0003	.0002
$MgSO_4$	.002	.002	.002	.002
$Mg(NO_3)_2$	...	.0035	.0037	.0038
Ca/Mg ratio	2/1	1/11	1/19	1/29

pH was adjusted to 5.5 using NaOH. Solutions changed weekly. B, Cu, Zn, Mn and Mo supplement added. Fe at 1 ppm added weekly as ferric tartrate.

Variations in the Ca/Mg ratios were obtained with equal osmotic pressures by substitution of $Mg(NO_3)_2$ for $Ca(NO_3)_2$. Serpentine species were not included in these experiments because it has not been possible as yet to grow them in culture solutions with consistent results. The yields and analyses of the leaf tissue of the buckwheat and sunflower are given in table V. The buckwheat grew fairly well even at the lowest Ca/Mg ratio in the culture solution, whereas sunflower died in this solution. The sunflower showed severe Ca deficiency symptoms even in solution B, whereas in this solution the appearance of buckwheat was normal. Buckwheat showed some Ca deficiency symptoms in both the C and the D solutions, however, in spite of the reasonably good growth.

Discussion

This study is concerned primarily with the differences which exist in the reactions of various species of plants to reciprocal changes in the exchangeable Ca and Mg of the soil, with special attention to the range in which the Ca/Mg ratio is less than 1/10. Such relationships are of particular interest in connection with serpentine soils, which are naturally low in Ca and high in Mg because of the parent material. Exchangeable Ca may make up as little as 5 % of the total cations in such soils. Even in serpentine soils in which exchangeable Ca is as high as 10 to 15 %, the data of Vlamis (15) and the present paper show that the yields of crop plants are severely restricted, and the infertility of such soils for agricultural production is well known. While other factors may contribute to this infertility, the low Ca level is believed to be the most limiting one. Native serpentine species are found to grow without restriction in yield on soils of 5 to 6 % Ca saturation, however, and may grow nearly as well on soils of less than 3 % Ca saturation (figs 2 and 3). At least one agricultural species, the common buckwheat, is able to tolerate Ca saturations as low as 5 % even though the yield is restricted (fig 3).

Table V

Yields and Composition of Sunflower and Buckwheat Grown in Solutions with Varying Ca/Mg Ratios

Solution	Average yield of shoot ** (gm dry wt)	Cations in leaf tissue (meq/100 gm dry wt)			
		Ca	Mg	K	Sum
Common sunflower (Helianthus annuus)					
A	5.48	55.8	18	139	213
B	1.69	15.9	48	126	190
C	0.31	13.2	65	141	219
D *	...	...	..	...	...
Buckwheat (Fagopyrum esculentum)					
A	3.12	64.7	50	75	190
B	2.25	17.1	60	58	135
C	2.05	10.5	52	68	131
D	1.42	8.5	87	66	162

* Plants died in this solution.
** Number of plants per jar: sunflower, 1; buckwheat, 5.

Differences which exist between plants in their ability to tolerate various types of soil media are well recognized, and crop plants differ widely in their absorption of cations even from the same soil (11, 13). Thus it is not surprising that they vary in their tolerance of low Ca levels, or as is actually the case here, low Ca/Mg ratios. Among the native plants studied there are also considerable differences in their reactions to the lowest Ca levels, as indicated by yield and by the appearance of obvious or incipient Ca deficiency symptoms. If many species were studied in detail, it should be possible to arrange them in a series with respect to their ability to grow and thrive in soils of low Ca/Mg ratios. Tomato, common sunflower, common buckwheat, *Streptanthus glandulosus typicus*, *S. g. pulchellus*, and *Helianthus bolanderi* constitute such a series from the plants used in this study, arranged in order of increasing ability to tolerate low Ca/Mg ratios in the soil. The physiological differences between these species which influence their abilities to absorb Ca and Mg would be of great interest but have been studied little as yet.

Analyses were made of the Ca, Mg, and K contents of the leaf tissue (tables II and III), expecting that these might offer some explanations for the differences in the reactions of the species studied to variations in the Ca–Mg status of the medium. *Helianthus bolanderi*, which is representative of the native serpentine species, absorbed appreciably more Ca at the low soil Ca levels than did a related species, the common sunflower. Furthermore, *H. bolanderi* absorbed much less Mg at the low Ca (high Mg) levels in Soil 30 than did the common sunflower. This effect in Mg absorption was seen in the Soil 85 experiments only at the lowest Ca (highest Mg) percentages. The tendency for "luxury" absorption of Mg by the crop plants from low Ca soil lots and the relative "exclusion" of Mg by the native species on these lots was nevertheless a trend in most experiments. Both the greater Ca absorption and the tendency to absorb smaller amounts of Mg by the native species result in a higher Ca/Mg ratio among the leaf tissue cations.

These analyses do not indicate clearly the reasons either for the greatly reduced yields of the crop plants at low Ca saturations in the soil or for the favorable growth of the serpentine species at these low saturations. Calcium and Mg concentrations in the leaf tissue roughly parallel the levels of these ions in the soil as would be expected. Whether an insufficiency of Ca, an excess of Mg, or a combination of these two effects limits yield in the crop plants cannot be stated with any certainty. The occurrence of typical Ca deficiency symptoms in these experiments at low Ca (high Mg) saturations, and the observation by Vlamis (15) that Ca deficiency symptoms could be induced in lettuce by additions of either Mg- or K-amberlites to a normal soil support the view that the insufficiency of Ca limits yield. The evidence which favors the belief that Mg plays only a passive role as a complementary ion is indirect and limited, however. The native serpentine species are able to absorb more Ca from soil containing low saturations of this ion, and often absorb less Mg at the same time. The Ca–Mg relationships in the leaf tissue of these species are thus more favorable. There appears to be no reason to assume that these plants have lower requirements for Ca than crop plants. Potassium absorption was relatively constant in spite of wide changes in the Ca/Mg ratio in the soil and in Ca and Mg contents of the leaf tissue. Fluctuations in the total cation contents of the tissue were too great to support well the concept of the "cation-equivalent constancy" (1).

A number of investigations, recently reviewed (9), have indicated that the Ca saturation of a soil needs to be only 20 to 40 % for good plant growth if the clay colloids are of the kaolinitic type, but must be considerably higher than this for optimum yields if the colloids are of the montmorillonitic type. On such a basis the serpentine soils used here, which gave high yields at Ca saturations of 25 to 30 %, would be expected to contain principally kaolinitic colloids. An x-ray diffraction pattern of the 2-μ fraction of Soil 30 indeed indicated that it was predominately kaolinitic.

Although serpentine soils were employed in this study, it is believed that the results obtained through their use are of broad application in considerations of Ca–Mg relationships. Other investigations have dealt largely with higher soil or solution Ca/Mg ratios in the range of 1/4 or larger, whereas the emphasis in these experiments is on the very low Ca/Mg ratios. The belief that data obtained using serpentine soils may be of more general use is supported by the fact that Vlamis (15) obtained similar yield curves either with a serpentine soil or with the non-serpentine Yolo loam in which he had reduced the Ca/Mg ratio to a series of low values by leaching with $CaCl_2$–$MgCl_2$ mixtures. Also in the solution culture experiments (table V), variations in the Ca/Mg ratio had effects similar to those noted in the soil experiments. Buckwheat was more tolerant of the low Ca/Mg ratios than the common sunflower, which developed some Ca deficiency symptoms and showed much reduced yield even in the B solution (Ca/Mg = 1/11), but the buckwheat grew moderately well in the D solution (Ca/Mg = 1/29).

We conclude that marked differences exist between species in their ability to tolerate low Ca/Mg ratios both in the adsorbed state in soils and in culture solutions. Certain species native to serpentine soils are able to tolerate especially low Ca levels, probably either because of unusually avid absorption of Ca or a low absorption of Mg, or a combination of these features.

Summary

By leaching with varying $CaCl_2$–$MgCl_2$ mixtures, lots of two serpentine soils were reconstituted into series ranging in percent exchangeable Ca from 2.8 to 82, with Mg as the complementary ion. Tomato, common sunflower, buckwheat, and three annual species native to California serpentine areas were grown on these soil lots in greenhouse pot cultures. In addition, sunflower and buckwheat were grown in culture solutions which ranged in Ca/Mg ratios from 2/1 to 1/29. Yields were recorded and the dry leaf tissue was analyzed for Ca, Mg, and K.

The principal results were as follows: 1) the yields of the crop plants were markedly depressed at exchangeable Ca levels of 20 % or less, and the plants made little or no growth at levels below 10 %; 2) the yields of the native species were not appreciably different within the range of 6 to 82 % exchangeable Ca, and were only moderately reduced in the 3 to 5 % range; 3) at the lower Ca levels, the native species absorbed more Ca and in most cases considerably less Mg than the crop plants; 4) the sunflower and buckwheat responded to the varying Ca/Mg ratios in the culture solutions in a manner similar to their reactions in the soil cultures.

It is concluded that the native species owe their greater tolerance of low Ca environments to their greater absorption of Ca and their usually lower absorption of Mg than the crop plants; there appears to be no reason to assume that the native serpentine species have lower requirements for Ca than agricultural plants. Although soils of serpentine origin were used in these experiments, the data are believed to be of general application in considerations of Ca–Mg nutrition.

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EFFECTS OF ULTRAVIOLET RADIATION AND CALCIUM AND THEIR INTERACTION ON SALT ABSORPTION BY EXCISED MUNG BEAN ROOTS[1,2]

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During the last few years, investigators studying salt absorption by plant roots have interpreted the selective uptake of ions by roots as being due to ion-binding carrier compounds with more or less specific binding sites. Overstreet and Jacobsen (11) have reviewed the various binding compounds proposed and have listed several characteristics which these compounds should have to fit into any working model of ion uptake. The recent proposal of Lansing and Rosenthal (7) identifying ribonucleic acid as a binding compound in Elodea cells has opened up several possible lines of investigation to test the role of nucleic acids in salt absorption by roots. One of these is the effect on salt absorption of ultraviolet radiation in the wavelength region of 2600 Å where there is strong absorption by nucleic acids. Several investigators have inferred that genetical effects of ultraviolet are due to absorption by nucleoproteins (8). Greenstein (6) has suggested that high sensitivity at 2600 Å be used to determine whether a biologically active material has nucleic acid associated with it. This line of investigation was carried out by studying the effect of ultraviolet radiation of 2537 Å on salt absorption by excised mung bean roots (*Phaseolus aureus*).

One of the salient features of salt absorption by roots is the stimulatory effect of polyvalent cations, which was first reported by Viets (13) for barley roots. Preliminary results have shown that salt absorption by mung bean roots is also greatly increased in the presence of Ca and Mg ions. The nature of this stimulation has been investigated. In addition, the suggestion of Viets (13) that the action of polyvalent cations is localized on or near cytoplasmic surfaces of root cells and the demonstration of ribonucleic acid on cell surfaces by Lansing and Rosenthal (7) have pointed to the desirability of looking for an interaction between radiation-induced effects and calcium ions on salt absorption.

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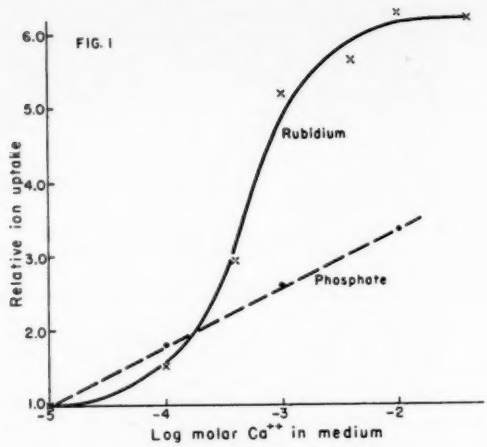

FIG. 1. Absorption of Rb and phosphate by excised mung bean roots as influenced by Ca concentration.

MATERIALS AND METHODS

Mung beans were germinated and grown at 25° C in contact with aerated 10^{-4} M $CaCl_2$ solution which was changed daily. Root tips 1 cm in length were excised from three-day-old seedlings. Fifty root tips were placed in a beaker, washed twice with deionized water, and placed in 50 ml solution of either RbCl or KH_2PO_4 of 10^{-4} M concentration containing tracer amounts of Rb^{86} or P^{32} (5 to 10 μc/l). The solution was vigorously aerated during the absorption period of 30 min at 25° C. After absorption, the radioactive solution was siphoned off and the roots rapidly washed 3 times with 10^{-2} M concentration of inactive carrier in 10^{-2} *N* HCl and 8 times in deionized water. The root segments were then placed in an aluminum dish, dried at 100° C, and the radioactivity assayed in the conventional manner.

In the ultraviolet studies, the root tips were irradiated with a General Electric 30 watt germicidal lamp at a distance of 30 cm. During the exposure period, the roots were in 50 ml of water and agitated by a magnetic stirrer. Absorption of rubidium and phosphate by control and irradiated roots was then determined in the absence and presence of 10^{-3} M $Ca(NO_3)_2$ or 10^{-3} M $MgSO_4$.

EXPERIMENTAL RESULTS

Before reporting the results, it might be pertinent to mention that preliminary studies have shown that with the concentration of salts used both Rb and phosphate uptake by excised mung bean roots were directly proportional to time up to two hours of absorption. Consequently, with an absorbing period of 30 min lack of substrates can be ruled out as a factor in these results. In order to conserve space, the results obtained with Mg have not been reported since they were essentially similar to those obtained with Ca.

EFFECT OF CALCIUM: Typical results showing Ca stimulation of Rb and phosphate uptake are presented in figure 1. Rubidium uptake was greatly enhanced by Ca and increased with increasing Ca concentration up to about 10^{-2} M Ca^{++}. Phosphate absorption was also stimulated markedly by Ca and increased with increasing Ca concentration. Incidentally, neither KNO_3 up to 10^{-2} M nor NaCl up to 10^{-2} M had any significant effect on phosphate uptake. The effects of high concentrations of K and Na on Rb absorption were not investigated because of the competitive nature of these ions on Rb absorption (4).

With the object of determining the locus of Ca stimulation, the experimental procedure was modified by reducing the time of absorption and increasing the specific activity of radiophosphorus. The shortest period of absorption employed was one minute. The remainder of the procedure was the same. Only phosphate uptake was studied in this manner. The findings obtained in these short-time studies are shown in figure 2. The results of phosphate uptake, with or without Ca, show a linear increase with time which appear to extrapolate to zero at zero time.

Because the preceding results suggested that mild treatments to remove Ca from roots might alter the course of salt absorption, excised roots were pretreated for one minute with dilute HCl and NaCl solutions and thoroughly washed with deionized water. Absorption studies were then carried out with these pretreated roots in the usual manner. Some of the results from these studies are presented in table I. Contrary to expectation, Rb uptake by pretreated roots was markedly increased by the HCl and NaCl treatments. On the other hand, phosphate uptake was decreased by the treatments. It can also be seen

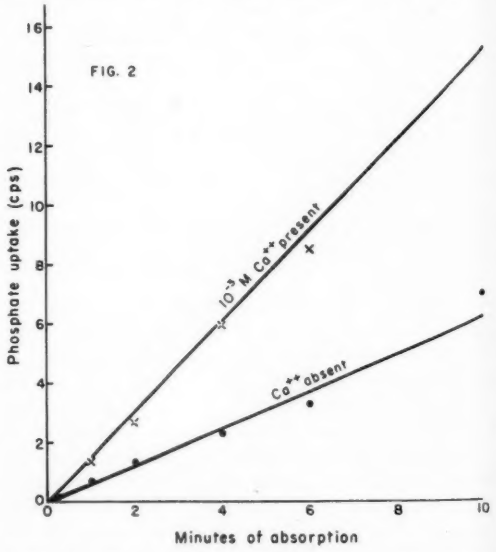

FIG. 2. Time course of phosphate absorption in the absence and presence of 0.001 M $Ca(NO_3)_2$.

that the Ca concentration employed reversed the effects of HCl and NaCl so that the treated roots behaved nearly like Ca-stimulated control roots. Magnesium also reversed the effects of HCl and NaCl. Other solutions of citric acid, ethylene diaminetetraacetate, and alkaline phosphate also increased Rb or decreased phosphate absorption.

ULTRAVIOLET EFFECTS: The effects of ultraviolet radiation on Rb and phosphate uptake are shown in figure 3. It can be seen from figure 3 that the response of excised mung bean roots to ultraviolet radiation is somewhat complicated. Irradiation increased the Rb uptake in the absence of Ca, but the presence of Ca brought about a severe reduction in Rb absorption. In contrast, phosphate absorption was reduced by irradiation, with Ca slightly enhancing the radiation effect. Somewhat similar results were obtained with Mg for both Rb and phosphate uptake. Potassium at a concentration of 10^{-2} M KNO_3, however, had no effect on phosphate absorption by irradiated roots.

A more detailed study of the effect of Ca on Rb uptake by irradiated roots was made by varying the concentration of Ca from 10^{-5} to 10^{-2} M as $Ca(NO_3)_2$. In this experiment, ultraviolet exposure was for two minutes. The results are shown in figure 4. Each point represents the relative Rb uptake by irradiated roots as compared to unirradiated roots in a medium of the same Ca concentration. According to figure 4, the relative ion uptake by irradiated roots appears to decrease linearly with increasing log Ca concentration.

DISCUSSION

Interpretation of the results reported here is made somewhat difficult by the complex nature of the influence of Ca and Mg ions on ion uptake by roots treated with ultraviolet radiation, HCl and NaCl

TABLE I

EFFECT OF PRETREATMENT WITH HCl AND NaCl SOLUTIONS ON SUBSEQUENT RUBIDIUM AND PHOSPHATE UPTAKE BY EXCISED MUNG BEAN ROOTS *

PRETREATMENT *	MOLAR Ca CONC IN ABSORPTION MEDIUM	RELATIVE ABSORPTION	
		RUBIDIUM	PHOSPHATE
None	0	100	100
"	0.0001	194	165
"	0.001	...	234
0.001 *N* HCl	0	468	74
"	0.0001	280	172
0.005 *N* HCl	0	...	47
"	0.001	...	225
None	0	100	100
"	0.0001	230	157
0.05 M NaCl	0	441	72
"	0.0001	307	158

* Excised roots were pretreated for one min and washed with water. Absorption was carried out in 0.0001 M RbCl or 0.0001 M KH_2PO_4 in the presence or absence of $Ca(NO_3)_2$.

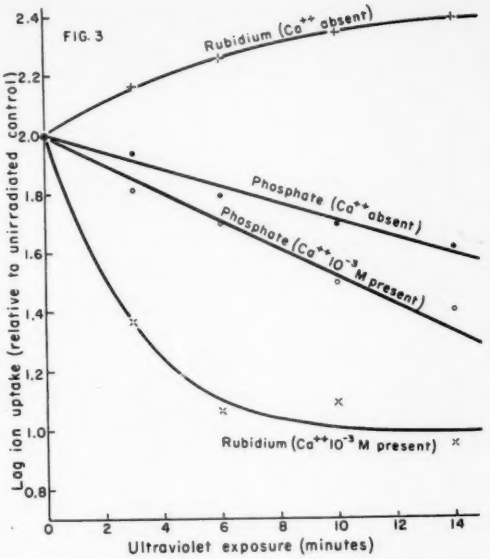

FIG. 3. Effect of ultraviolet radiation on Rb and phosphate uptake by excised mung bean roots. After irradiation, absorption was conducted in the absence and presence of $Ca(NO_3)_2$.

solutions. The rates of both Rb and phosphate absorption by mung bean roots are greatly increased in the presence of Ca and Mg (fig 1). The magnitude of stimulation is much greater than similar Ca-stimulated K uptake reported earlier by Viets (13) for barley roots. Overstreet et al (12) have postulated that Ca enhances K uptake by acting as a cofactor in the breakdown of a K complex, KR, where R is a binding compound.

From the results presented in figure 2, which show the absence of any noticeable lag in the Ca-mediated stimulation of P uptake, and in view of the known slow uptake of Ca, it appears that the locus of action of Ca ions might be located on or near the external surface of the cytoplasm of epidermal cells. Viets (13) has made the same suggestion. Further evidence in support of a surface reaction was obtained in an experiment in which the results indicated that Ca stimulation occurred only when it was present in the medium during absorption. In this experiment excised mung bean roots were pretreated for 30 min in a 10^{-3} M $Ca(NO_3)_2$ solution and thoroughly washed with water. Absorption of radiorubidium and radiophosphorus in the absence of Ca by these roots amounted to 7.6 and 6.4 cps, respectively. On the other hand, roots not pretreated with Ca absorbed four times more radiorubidium and 2.7 times more radiophosphorus in the presence of 10^{-3} M $Ca(NO_3)_2$.

Besides its role in stimulating both anion and cation uptake, Ca appears to play an additional part during salt absorption by mung bean roots. The reversal by Ca of effects brought about by acid and

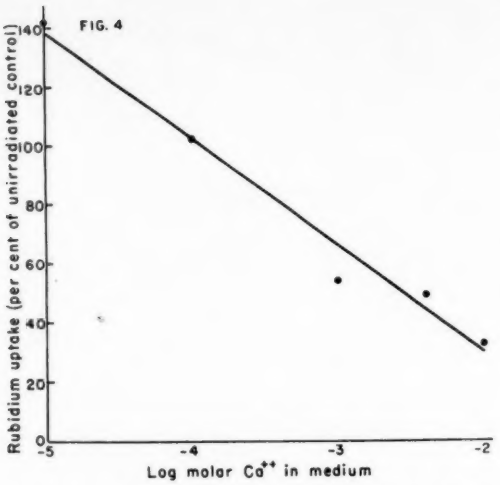

FIG. 4. Effect of Ca concentration on Rb uptake by excised mung bean roots irradiated with ultraviolet. Radiation exposure was for 2 min. Each point is the relative Rb absorption of irradiated roots as compared to control roots in a medium of the same Ca concentration.

NaCl treatments (table I) indicates that these treatments removed Ca ions and that Ca plays some part in regulating cation and anion uptake. At present no adequate explanation is proposed as to how Ca "regulates" salt absorption.

The action of Ca on salt absorption by irradiated roots (figs 3 and 4) points to a strong interaction between radiation effects and Ca ions. Here also, the response of irradiated roots to Ca in the external medium suggests that the site of ultraviolet-Ca interaction is located on or near the cytoplasmic surface of the epidermal cells. Such an interpretation leads one to consider the possibility of a close connection between the salt-absorbing system acted upon by polyvalent cations and the radiation-sensitive system.

Since relationships which show a linear dependence of "log survival fraction" with dose have been interpreted as due to "one-hit" inactivation (8), some of the results presented in figure 3 could also be interpreted in the same manner. The data of phosphate absorption by irradiated roots appear to show the operation of such a "one-hit" mechanism. Likewise, the reduction of rubidium absorption by irradiated roots in the presence of calcium at the lower ultraviolet exposures also suggests a "one-hit" mechanism. Rubidium absorption by roots exposed to the higher ultraviolet exposures, which amounts to about 10 % of the control, could be due to exchange reactions.

Obviously, if the identity of ion-binding carrier compounds involved in salt absorption were known, interpretation of the results reported here would be facilitated. However, in view of our ignorance as to the nature of such compounds, one can only speculate and propose some likely compounds. Owing to their strong absorption of radiation at 2537 Å, the purine- and pyrimidine-containing nucleosides, nucleotides, and polymers are the most likely to be affected by radiation of that wavelength. From the chemistry of the simple nucleosides and nucleotides, it is difficult to visualize how specificity of cation- and anion-absorption sites (3, 4) can be attributed to them. This tends to rule out the possibility of their being the ion-binding compounds. The complex polymers, such as the nucleic acids, offer greater possibility of having specific absorption sites. Moreover, action spectrum studies of biological effects of ultraviolet radiation have indicated that in many instances nucleic acids are the immediate receptors of the effective radiations in the neighborhood of 2600 Å (5). The observation of Lansing and Rosenthal (7) that Elodea cells treated with ribonuclease lose their capacity to absorb Ca strongly indicates that ribonucleic acid participates in some manner in salt absorption—probably as an ion-binding compound as they have suggested. If ribonucleic acids, or more likely ribonucleoproteins, are ion-binding compounds, the effect of ultraviolet on salt absorption could be due to changes brought about in them by the radiation. Some probable changes which might explain some of the reported results could be unfolding of coiled molecules and greater sensitivity to salt denaturation (5, 9). If ribonucleoproteins do take part in salt absorption, the negative charge of the nucleic acid would enable it to bind cations, while the positive charge of the protein moiety would bind anions.

Although the results do not show unequivocally the direct involvement of a ribonucleoprotein in salt uptake, other observations can be mentioned which support the proposal that a ribonucleoprotein could be a salt-binding carrier compound in the initial uptake of salts by plant cells. Besides their work with ribonuclease, Lansing and Rosenthal (7) have reported cytological observations which show a localization of ribonucleic acid on surfaces of cells. Claude (2) has pointed out that ribonucleic acids are often found associated with phospholipids, the latter probably forming the cell membrane. The hydration of nucleoproteins has been shown to be markedly affected by polyvalent cations (1). This property might be a clue leading to the explanation of the stimulation of salt absorption by polyvalent cations. Neuberg and Roberts (10) have shown that nucleic acids have remarkable properties to form complexes with many insoluble salts, and they have pointed out that the reactive groups on a nucleic acid molecule can form numerous combinations. The different ion-binding potentials of the reactive groups and their positions on nucleic acid and protein molecules could probably explain the specificity of cation- and anion-binding sites as reported by Epstein and Hagen (4) and Epstein (3). Since it is highly probable that ribonucleoproteins from various species of plants would show some differences in properties, some of the differences in the nature of salt absorption among

plants could be due to variations in the ion-binding properties of their ribonucleoproteins.

SUMMARY

The effects of Ca, ultraviolet radiation, dilute HCl and NaCl solutions on salt absorption by excised mung bean roots (*Phaseolus aureus*) were investigated using Rb^{86} and P^{32} as tracers. Both Rb and phosphate uptake were greatly enhanced by the presence of Ca. Short-time studies indicated that the locus of Ca action is on or near the outer surface of the cytoplasm. Short periods of pretreatment of excised roots with dilute HCl and NaCl solutions increased subsequent Rb uptake but decreased phosphate absorption. The effects of HCl and NaCl were reversed by Ca.

Rubidium absorption was increased by ultraviolet radiation of 2537 Å, but the presence of Ca during absorption drastically decreased Rb uptake by irradiated roots. Phosphate absorption by irradiated roots was reduced by ultraviolet radiation to a slight degree, and was further decreased by the presence of Ca ions. Some of the absorption curves show a linear decrease of log ion uptake with ultraviolet dose.

The results obtained have been interpreted as being due to the involvement of a ribonucleoprotein in salt absorption. The suggestion has been made that the nucleic acid binds cations, while the protein moiety binds anions.

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ON THE NATURE OF THE ENZYMATICALLY CATALYZED OXIDATION PRODUCTS OF INDOLEACETIC ACID [1]

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The plant growth hormone, indoleacetic acid (IAA), is known to be oxidatively inactivated by enzyme preparations from various plant sources (6, 12, 16, 18, 19). It has usually been assumed that the oxidation product is 3-indolealdehyde (16, 19). The evidence for this belief is as follows: (a) Manometric and colorimetric measurements made during the oxidation show that one mole of O_2 is absorbed and one mole of CO_2 is released per mole of IAA disappearing (16, 19); (b) The product yields colors with Ehrlich's reagent (19) and with the Hopkins-Cole reagent (16) which could be due to the persistence of the indole ring; (c) The product apparently has a carbonyl group, since it forms an insoluble 2,4-dinitrophenylhydrazone (19).

Our reinvestigation of this question was prompted by several facts. In the first place, other products may be proposed, the properties of which reasonably fit all of the known data (fig 1). Secondly, the apparent similarity of the IAA oxidase system from etiolated peas to tryptophan oxidase from various sources (9, 10, 20) suggested a mechanism of oxidation involving cleavage of the indole ring. Thirdly, neither Ehrlich's reagent nor the Hopkins-Cole reagent provides a specific test for the indole ring, certain aromatic amines giving positive reactions with the former, and certain indolic compounds failing to give a pronounced color with the latter.

We therefore undertook to characterize the products of the oxidation of IAA by the IAA-oxidase of etiolated peas, using paper chromatography as a diag-

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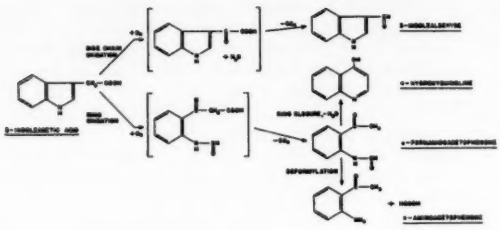

FIG. 1. Possible oxidation pathways of indoleacetic acid involving a 1:1:1 stoichiometry of IAA destroyed : O_2 absorbed : CO_2 evolved.

nostic tool. The results are definitive in ruling out 3-indolealdehyde, *o*-formamidoacetophenone, *o*-aminoacetophenone and 4-hydroxyquinoline as major end products of the oxidation. The data also permit the formulation of other compounds which may in fact result from this oxidation.

MATERIALS AND METHODS

PREPARATION AND FRACTIONATION OF THE CRUDE ENZYME EXTRACT: Alaska peas were planted in flats of moist vermiculite and allowed to germinate and grow in a darkroom maintained at $25 \pm 1°$ C for a period of 7 to 9 days. The epicotyls were harvested, cut into small sections, and ground with minimal volumes of Sørensen's phosphate buffer, pH 6.6, in a Waring blendor at 0 to 2° C. The resulting brei was passed through cheesecloth to remove the larger particles and the filtrate was centrifuged in the cold for 30 minutes at $10{,}000 \times g$. The milky supernatant fluid was then stored in a deep-freeze until it was further purified by the stepwise addition of solid reagent grade ammonium sulfate. The ammonium sulfate precipitations occurred over periods of 5 to 12 hours, after which the various precipitates were collected by centrifugation at $10{,}000 \times g$ for 20 to 30 minutes. They were then resuspended in phosphate buffer, pH 6.6, and stored in the refrigerator at 0 to 2° C. Such preparations showed no appreciable loss of activity when stored for a period of at least two weeks.

The IAA-oxidase activity of each fraction was determined by incubation of a 1-ml aliquot with 3-indoleacetic acid (Eastman) at an initial concentration of 130 μg/ml for an interval of ca 65 minutes, after which the remaining IAA was determined by the colorimetric method with the Salkowski reagent as described by Tang and Bonner (16). In every case, the reaction mixture contained 10^{-5} M 2,4-dichlorophenol as cofactor (5).

Although all fractions were found to have considerable activity, highest activity was usually found in the final supernatant liquid remaining after complete saturation with ammonium sulfate. This fraction was used in most of the oxidation experiments. In several preliminary experiments, we obtained indications that all of the fractions yielded similar oxidation products, so that no further purification of the enzyme was attempted in these studies.

OXIDATION EXPERIMENTS: In a typical preparative oxidation, 50 to 100 ml of an aqueous solution of IAA at an initial concentration of 500 μg/ml were mixed with an equal volume of the enzyme solution in a 500-ml Erlenmeyer flask, and the mixture incubated at room temperature with gentle shaking on an Eberbach shaker. The oxidations were allowed to proceed to about 75% completion (ca 2 hours) as determined by colorimetric assay of withdrawn aliquots for IAA. At the end of this time the products of the reaction were extracted with reagent grade chloroform. This extract was dried and concentrated in vacuo to a few ml volume.

CHROMATOGRAPHY OF THE PRODUCTS: Chromatographic separation of the oxidation products was performed upon ascending chromatograms of Whatman No. 1 filter paper, by the use of conventional techniques (2, 15).

Two mixtures containing isopropanol, concentrated aqueous ammonia, and water were used as developing solvents. With a 10:1:1 volume ratio (2) the solvent front moved rapidly, nearing the upper end of the cylinder in 6 hours. A 2:1:1 volume ratio, however, proved superior for the separation of the oxidation products and was used in the development of most of the chromatograms. This developer moved slowly, 15 hours being usual for development.

Several spray reagents were used. A solution of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde), 1% in 1 *N* hydrochloric acid, was found to be an effective color reagent for indole and aromatic amine-type compounds. A solution of ferric chloride, 1% in water, was used as a spot reagent for 4-hydroxyquinoline and was later found to give color reactions with some of the other compounds involved. The Hopkins-Cole reagent, prepared according to Benedict (1) was also employed, as follows: To a small amount of the material to be tested were added one ml of the reagent and 0.2 ml of 0.01 M aqueous cupric sulfate. Five ml of concentrated sulfuric acid were added cautiously and finally mixed thoroughly with the solution.

REAGENTS: Eastman indoleacetic acid was used without further purification. 3-indolealdehyde was prepared by Mr. R. A. Bernhard of these laboratories. It was crystallized twice from water to give white blades, insoluble in aqueous sodium hydroxide. The melting point was 193.5 to 195° C, which agrees well with the previously cited figure of 194° C (11). *o*-Formamidoacetophenone was prepared by the method of Witkop (21). Recrystallization of the product from ether containing a small amount of ligroin gave sparkling prisms. Their melting point of 78.9 to 79.5° C agrees well with the literature value (21) of 78° C. *o*-Aminoacetophenone was prepared by refluxing 0.25 gm of crude *o*-formamidoacetophenone with 20 ml of 3 *N* hydrochloric acid for 45 minutes. The cooled mixture was neutralized by the addition of 5 to 6 gm of sodium bicarbonate, which caused the separation of *o*-aminoacetophenone as a dark oil. The oil was co-

distilled from the neutral mixture with water and separated from the latter by ether extraction. Removal of ether left the *o*-aminoacetophenone as a yellow, pleasant smelling oil. 4-Hydroxyquinoline was prepared according to the procedure of Camps (3). The product had a melting point of 198 to 205° C, which is in the range of the previously published figure of 201° C (3). The product gave an orange to red color with $FeCl_3$ in aqueous solution, which also agrees with the reported behavior of this substance.

Experimental Results

Color Reactions of Known Compounds: Table I summarizes the average R_f values of various known compounds and their color reactions with the different reagents. The fact that Hopkins-Cole reagent gives a pale yellowish color with 3-indolealdehyde indicates that this compound alone could not have accounted for the purple color observed by Tang and Bonner (16). With the other indolic substances tested, this reagent gave various shades of red and purple. Apparently the presence of the indole nucleus is a necessary, but not sufficient, condition for an intense color reaction with this reagent.

With respect to Ehrlich's reagent, the compounds tested fall into two general categories. Compounds containing the indole nucleus gave colors ranging from pink to blue-violet after at least several minutes of development time. Compounds containing an aromatic amino group, on the other hand, give an immediate color with Ehrlich's reagent due to Schiff base formation. Thus, *o*-aminoacetophenone (OAA) and *o*-formamidoacetophenone (OFA) both give immediate intense orange-yellow coloration, this color persisting indefinitely in the case of OAA and changing slowly to blue in the case of OFA. Knox and Mehler (9) found kynurenine to give a similar yellow color with Ehrlich's reagent.

Hopkins-Cole tests were carried out both on the reaction mixture and on a chloroform extract of the reaction mixture previously adjusted to pH 9.0. Blank determinations upon an enzyme solution were also performed. When performed directly upon a reaction mixture, the Hopkins-Cole test gave a pinkish-brown color regardless of the presence or absence of IAA or its oxidation products. This color probably results from the high "blank" of tryptophan-containing proteins in the enzyme preparation. The oxidation reaction mixture also contained a substance, presumably indolic, which could be extracted into chloroform and which gave a Hopkins-Cole color after removal of the chloroform. This was shown to be neither present in, nor formed from the enzyme preparation alone. That it was not unchanged IAA was indicated by a negative Hopkins-Cole test given by a chloroform extract of an IAA solution at a comparable concentration and pH 9. This Hopkins-Cole positive substance, so easily extracted into chloroform, was not further identified in any of the paper chromatograms described below.

Chromatographic Separation of the Oxidation Products: Many paper chromatograms from the chloroform extract of eight oxidation experiments indicated the presence of at least two principal oxidation products. These appeared, before any spray treatment, as yellowish spots having average R_f values of 0.94 and 0.91, respectively. Upon spraying with Ehrlich's reagent, both spots were immediately colored orange-yellow, resembling OFA and OAA in color and behavior. Upon standing, however, both spots, unlike either OFA or OAA, began to develop reddish tinges, the spot at R_f 0.94 becoming pinkish-orange and the lower one dark pinkish-orange to light red. These colors were dependent upon the concentrations of the oxidation products and deepened toward red with increasing concentration. That this color ripening did not result from a superposition of an aromatic amine and IAA was indicated by the presence of IAA as a clearly defined violet spot below the oxidation products.

Treatment with ferric chloride spray revealed both products as rose colored spots, which appeared within 3 to 5 minutes and intensified with time, particularly the R_f 0.94 spot which became deep rose upon standing. This is unlike the behavior of 4-hydroxyquinoline which gives an immediate and unchanging orange spot

Table I

R_F Values and Color Reactions of IAA and Possible Products of Its Oxidation

Substance	R_F		Color development		
	10:1:1 Developer	2:1:1 Developer	Hopkins-Cole	Ehrlich	$FeCl_3$
IAA	0.35	0.79	Violet	Violet in ca 5 min	Reddish (slow)
3-Indolealdehyde	0.90	0.91	Pale yellow	Violet in ca 10–15 min	Reddish (very slow)
OFA	0.87	0.92	None	Immediate orange-yellow, becomes bluish on standing	None
OAA	0.89	0.93	None	Immediate orange-yellow	None
4-Hydroxyquinoline	...	0.83	.....	None	Immediate orange
Skatole	0.87	...	.....	Blue-violet in 5 min	.....
Indole	0.92	...	.....	Pink in ca 5 min	.....
Kynurenine	...	...	.....	Yellow	.....
Anthranilic acid	...	...	.....	Immediate orange-yellow	Pale yellow (slow)

TABLE II

ACCUMULATION OF THE OXIDATION PRODUCTS WITH TIME

SPOT	R_F	COLOR DEVELOPED WITH EHRLICH REAGENT	RELATIVE COLOR INTENSITY (I) AFTER VARYING PERIODS (MIN) OF REACTION
IAA	0.80	Violet in 5 min	$I_{65} >> I_{100} > I_{270}$
Oxidation product I	0.91	Yellow→Red	$I_{65} < I_{100} << I_{270}$
Oxidation product II	0.94	Yellow→Orange	$I_{65} < I_{100} < I_{270}$

with ferric chloride. This and the fact that 4-hydroxyquinoline gives no color with Ehrlich's reagent eliminate it as the product responsible for the observed spots. Beside failing to give Ehrlich tests that correspond to those of the oxidation products, 3-indolealdehyde, OFA and OAA behave differently with ferric chloride as well. OFA and OAA give no color with this reagent even upon standing, and 3-indolealdehyde gives only a faint reddish spot upon standing 10 to 20 minutes (table I).

To confirm the identity of the above spots as authentic reaction products, and to detect any possible change in the nature of these products or in the approximate ratios of their concentrations with time, an experiment was performed in which one-third of the reaction mixture was withdrawn at each of three successive time intervals and extracted with chloroform to remove the oxidation products. The results of paper chromatography of these chloroform extracts are indicated in table II. With each aliquot, three spots appeared upon treatment with Ehrlich spray. An upper pair, at R_f values of 0.94 and 0.91, appeared immediately as yellow spots which ripened either to orange (R_f 0.94) or bright red (R_f 0.91). Residual IAA appeared as a violet spot at R_f 0.80. It was clear that intensities of the two upper spots, and in particular, the one at R_f 0.91, increased in the same order. The same results were obtained with ferric chloride as a spray reagent. This indicates that as IAA is destroyed, at least two oxidation products accumulate in the reaction mixture. The faster-moving component is extracted by chloroform from reaction mixtures adjusted to either pH 3.0 or 9.0; the slower-moving component is extracted only at pH 3.0.

ULTRAVIOLET ABSORPTION SPECTRA OF THE OXIDATION PRODUCTS: To ascertain the possible presence of oxidation products not responding to either of the spray reagents employed, the paper chromatograms were cut into strips representing various R_f's, the strips eluted with methanol, and the absorption of the eluate measured in a Beckman spectrophotometer at 220 mμ, 250 mμ and 280 mμ. In figure 2, the optical densities at these three wavelengths are plotted versus the R_f values corresponding to the strips. It appears that there are no major products with marked absorption in the ultraviolet which do not also respond to the spray reagents used.

The product of R_f 0.94 extracted at pH 9 possesses an absorption maximum at 280 mμ and a minimum at 255 mμ (fig 3). In contrast, the material responsible for the R_f 0.94 spot extracted at pH 3 lacks the 255 mμ minimum and the maximum at 280 mμ. This difference is probably due to the presence of a second product in one of the R_f 0.94 fractions whose spectrum is superimposed upon that of a common product. Although the spectrum of the material extracted at pH 9 resembles that of the added cofactor, 2,4-dichlorophenol, the concentration of this substance which might be present in the final methanol solution (at the most, 3×10^{-5} M) is insufficient to account for the observed optical densities.

OFA AND 3-INDOLEALDEHYDE AS POSSIBLE OXIDATION INTERMEDIATES: Although it appeared that the products of the oxidation were not identical with either OAA, OFA or 3-indolealdehyde, the possibility remained that one of these compounds was formed initially from IAA and subsequently converted into other products. To test this possibility, OFA and 3-indolealdehyde were incubated with preparations of IAA-oxidase in separate experiments. The conditions employed were the same as previously described. In each experiment the concentration of the substrate was comparable to the initial concentrations of IAA used in the other experiments, and chloroform extraction was performed at the pH of the reaction mixture following the incubation period. The Ehrlich's

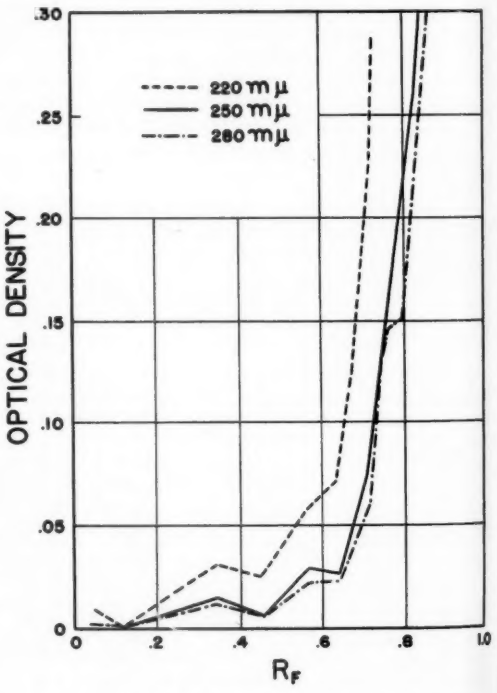

FIG. 2. Distribution of ultraviolet-absorbing materials on the chromatogram.

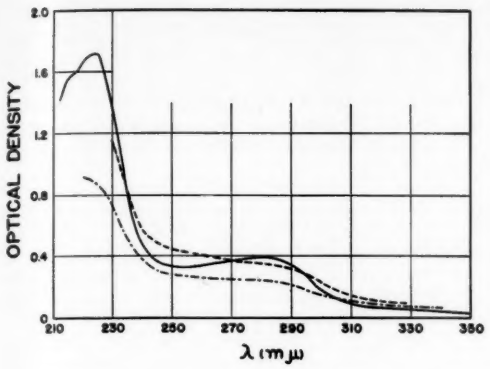

FIG. 3. Ultraviolet absorption spectra of oxidation products of IAA.

——— R_f 0.94, extracted at pH 9.0
------ R_f 0.94, extracted at pH 3.0
-·-·-·- R_f 0.91, extracted at pH 3.0

reagent and ferric chloride treatment of the chromatograms made from the chloroform extracts indicated clearly that neither OFA nor 3-indolealdehyde was acted upon by the oxidase. Thus, neither can be considered an intermediate in the formation of the observed oxidation products.

DISCUSSION

The evidence obtained in this investigation indicates that when IAA undergoes oxidative degradation in the presence of the IAA-oxidase system, it gives rise to several oxidation products. At least one of these products contains an indole ring, as suggested by the red Hopkins-Cole color given by a chloroform extract of a reaction mixture. That this color *does not* result, however, from the presence of 3-indolealdehyde as was originally proposed (16, 19), is shown by the failure of samples of the aldehyde to give a similar color when treated with Hopkins-Cole reagent. At the same time, there is evidence (immediate formation of an orange-yellow color with Ehrlich's reagent) that at least two of these products contain aromatic amino groups, these necessarily resulting from cleavage of the indole nucleus.

Although OFA, OAA, 3-indolealdehyde and 4-hydroxyquinoline all possess R_f's within the range of the oxidation products, their behaviors with the spray reagents employed differ from those of the actual oxidation products. A similar conclusion has apparently been reached by other workers, using an enzyme from the fungus *Omphalia flavida* (14).

While the present indications that the indole ring of IAA is largely destroyed are in opposition to the conclusions of previous workers (16, 19), some of their data favor the concept of ring opening. The destruction of the indole ring of tryptophan reported by Knox and Mehler (9) and by Wiltshire (20) has already been mentioned. Wagenknecht and Burris (19) report that whereas the enzymatic oxidation of IAA results in the liberation of one molecule of carbon dioxide per molecule of oxygen consumed, the oxidation of 3-indolepropionic acid occurs with an RQ of only 0.20. Such a reduced yield of carbon dioxide in the case of 3-indolepropionic acid could be explained by an opening of the indole ring to give a gamma-keto acid, which would be more resistant toward decarboxylation than the corresponding beta-keto acid resulting from IAA. Although the liver tryptophan oxidase studied by Knox and Mehler (9) was specific for the naturally occurring L-isomer, the pea seedling oxidase employed by Wiltshire (19) (possibly identical with our IAA oxidase) brought about the oxidation of both D- and L-tryptophan at the same rate. It is reasonable that such behavior could result from an enzyme-substrate complex involving attachment via the indole nucleus alone, and not involving the side chain which contains the optically active carbon atom.

It should be noted that other possible explanations exist for the observed stoichiometry of the reaction. For example, the formation of any isomeric dihydroxy skatole would occur with the uptake of one molecule of oxygen and the liberation of one molecule of carbon dioxide per molecule of IAA inactivated. A compound of this type could not, of course, give a Schiff's base color with Ehrlich's reagent, and so is also excluded as a product.

Barring OFA (or anything arising from it with no further gas exchange) as an oxidation product, it is difficult to devise a hypothetical scheme involving ring opening and in harmony with the observed 1 : 1 : 1 stoichiometry. The present experiments have demonstrated, however, that the IAA-oxidase catalyzed degradation of IAA leads not to one, but to several products. It therefore appears unnecessary to attempt to formulate a single product capable of explaining the stoichiometry when the net gas exchange observed is certainly the resultant of a number of degradation pathways. Such a multiple pathway for the destruction of IAA is hardly surprising in view of the known complexity of the enzyme system involved and it is particularly suggested by the fact that IAA plays a dual role in the enzymic sequence. Not only is it the substrate for the H_2O_2-peroxidase complex (4), but it may also react to produce peroxide for its own destruction (13).

If the stoichiometry of the inactivation process need not be accounted for by the formation of a single substance it becomes possible to propose other reasonable products resulting from oxidative cleavage of the indole nucleus. One such possibility involves, in addition to opening of the pyrrol ring, hydroxylation of the benzene ring. Such a hypothesis derives some support from the apparent formation of 3-hydroxykynurenine and 3-hydroxyanthranilic acid by enzymes from pea seedlings (19), rat (8) and Neurospora (7). If a hydroxy OFA (or OAA) were to arise from the IAA-oxidase-mediated degradation of IAA, the hydroxylation step would necessarily (since OFA is known not to be an intermediate) occur at

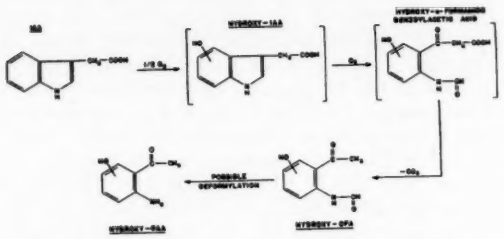

FIG. 4. A proposed reaction sequence and end product accounting for all the observed data for one product of IAA oxidation.

some point before the actual ring opening. A final product such as hydroxy OAA (fig 4) would appear to satisfy all the requirements for the observed oxidation product. It would be capable of giving a reaction with Ehrlich's reagent and probably, by virtue of the phenolic hydroxyl, a color with ferric chloride as well.

SUMMARY

In the presence of pea seedling IAA-oxidase, IAA undergoes oxidative conversion into at least two and probably three products. Application of chromatographic techniques and color reagents indicated that some of the more obvious hypothetical products cannot account for the observed behavior of the authentic oxidation products. The compounds ruled out as major end products were 3-indolealdehyde, *o*-formamidoacetophenone (OFA), *o*-aminoacetophenone (OAA) and 4-hydroxyquinoline. It is also found that neither OFA nor 3-indolealdehyde participates as intermediates in the formation of the final oxidation products.

Color reactions with Ehrlich's reagent and with ferric chloride further indicate that the two most prominent oxidation products contain aromatic amino groups and phenolic hydroxyl groups. At the same time, the presence of a third compound containing an indole ring is suggested by a positive Hopkins-Cole reaction of a chloroform extract of the reaction mixture.

While the behavior of OFA with the various color reagents employed is not identical with the behavior of the actual oxidation products, there are close enough similarities to warrant the assumption that some of these products are close to OFA in structure. It is suggested that the neutral oxidation products of R_f 0.94 in 2 : 1 : 1 isopropanol-ammonia-water could be a hydroxy-*o*-aminoacetophenone, which may arise by hydroxylation of the beneze ring, followed by oxidation of the pyrrol nucleus.

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IDENTIFICATION OF THE CARBON DIOXIDE BURST IN CHLORELLA USING THE RECORDING MASS SPECTROMETER [1,2]

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The problem of whether the assimilatory quotient remains constant throughout the period immediately following the onset of illumination has been frequently discussed both from the experimental and theoretical point of view. The importance of this problem has been particularly emphasized with reference to determinations of the quantum efficiency of photosynthesis of Chlorella during brief light periods. Warburg and Negelein (11) in their earliest experiments determined the assimilatory quotient by gas analysis and used these values to justify their manometric observations. Emerson and Lewis (5) subsequently pointed out that this procedure was not justified because the period in which quotient measurements were obtained was much longer than that which was used for measuring photosynthetic efficiency. Further, Emerson and Lewis, using rapidly shaken differential manometers, were able to show that, during short periods of alternate light and dark, unexpected transients in pressure change occurred. Using the well known equations developed for the "two-vessel" method, they concluded that these pressure changes were to be attributed to a "burst" or rapid production of CO_2 immediately after the light was turned on and a corresponding "gulp" or uptake of CO_2 after the light was turned off. Thus, the earlier estimates of quantum efficiency made by Warburg and Negelein, in which it had been assumed that no abnormal transients occurred, were made doubtful.

The conclusions of Emerson and Lewis were consistent with earlier observations by Blinks and Skow (1) who had observed rapid changes in potential of a glass electrode placed in close contact with an illuminated Castalia leaf. Similar results were obtained using leaves of Potamogeton and some land plants. These workers reported an "acid" gush which may be identified with the CO_2 burst subsequently discovered in Chlorella.

Warburg was unwilling to accept the suggestion that the CO_2 burst called for reinterpretation of his previous quantum yield measurements. He belittled its importance (12). Within the past few years Warburg and coworkers, using manometric techniques, have reported numerous measurements whereby they have attempted to resolve O_2 and CO_2 exchanges over short time intervals in successive brief periods of illumination and darkness (3). In none of these studies has a CO_2 burst been reported.

No other investigator has attempted to duplicate or improve upon the original measurements of Emerson and Lewis. Using quite different methods, Van der Veen (9) observed a CO_2 burst in Chlorella spread on a wet piece of filter paper. But there remained the need for observations by a method specific for CO_2, and under conditions essentially the same as those previously used for quantum yield determinations. This led to the present work.

The manometric method, used by Emerson and Lewis, is necessarily unspecific, since observations are made solely of pressure changes. From the observed pressure changes in two vessels, the calculation of the amounts of different gases exchanged depends on certain assumptions: first, that only two gases are undergoing partial pressure changes, second, that they differ markedly in solubility, and third, that the physical response of each manometer follows faithfully the net changes in the concentrations of dissolved gases (allowing, of course, for their solubilities). This third assumption is valid if the system is in all respects in a steady state or if transitions from one steady state to another are relatively slow compared with the response of the measuring system to changes in dissolved gas concentrations. Conversely, if the transitions are relatively rapid, manometric detection of increments in the concentrations of dissolved gases cannot be considered quantitatively reliable since differences in half-time for manometric response may differ between vessels and between gases. Moreover, the validity of the first of the above assumptions is not immediately apparent. Gaffron (6) has shown with some green algae, e.g., Scenedesmus, a photochemical metabolism of hydrogen; while this metabolism is absent in Chlorella in the steady state, its possible existence during brief transitory periods remains to be investigated. The experiments of Emerson and Lewis may also be criticized on the grounds

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that the two different vessels were not observed simultaneously; the observations were, in fact, separated by a time interval of several hours.

In the present investigation a mass spectrometer was used to analyze the gas phase over the algal suspension in the reaction vessel. The latter was a rectangular Warburg flask shaken in a constant temperature bath in the same manner as the conventional Warburg manometer assembly. The method permits, under conditions duplicating those of conventional manometry, the specific identification in a single vessel of the gas or gases responsible for the observable pressure changes. With suitably cultured algal cells, a burst of CO_2 was observed at the onset of illumination. Other gases (CO, H_2, O_2, N_2) were shown not to be significantly involved in the pressure burst. The characteristics of such bursts (their dependence upon experimental conditions), were found to be substantially as reported by Emerson and Lewis.

Materials and Methods

Chlorella pyrenoidosa (Emerson's strain) was maintained in pure culture. For experimental purposes it was grown autotrophically in liquid medium of the composition described by Emerson and Lewis (4). A continuous stream of air, enriched with 5 % CO_2, was bubbled through the cultures. Cells were illuminated by either fluorescent lights (5×20 watt white strips 15 cm away) or by tungsten light (25 cm from a strip of 100-watt bulbs). In order to maximize the burst which the cells subsequently exhibited, the temperature of the culture bath was varied; 12 to 15° C was found most favorable. Emerson and Lewis recommended reduction of light intensity during the last day or two of culture as a means of enhancing the CO_2 burst. This was done in nearly all our experiments by the substitution of 40-watt bulbs for the 100-watt bulbs. In some experiments cells grown at 25° C were placed at 5° C and the light intensity reduced 12 hours before measurement. Cells from such cultures frequently exhibited conspicuous bursts. In other experiments cells were grown at 25° C for 4 days at the full intensity and then for a further 2 days at reduced intensity; at 15° C, the growth period was 6 days at full intensity and 2 further days at reduced intensity. The density of the culture at the time of harvest was of the order of 2 μl per ml. With cells grown in fluorescent light at both 15° and 25° C, the temperature was found to have less effect than in the case of cells grown in tungsten light. This was true also of the effect of reducing the light intensity for the final period of growth.

The manner in which the mass spectrometer (Consolidated Engineering Company Model 21-201) was adapted for metabolic studies has been explained in detail (2). The incorporation of the mass spectrometer leak into a male joint, to which the reaction flask is attached, has also been described (8). The only further modification in the apparatus which this study necessitated was reconstruction of the gas inlet system in the region of the ion source. In the spectrometer as supplied commercially the gas was admitted to the source through tubing of very small bore which made the pump-out time for gas entering from the leak unnecessarily prolonged. The half-time for spectrometer response was about 20 seconds and this limited the time resolution of the measurements. After replacing all small-bore tubing, so that a 0.64 cm (0.25 inch) I.D. passage was available at all points between leak and ion source, the pump-out half-time of the spectrometer tube was reduced to about 2 seconds. The time resolution of metabolic rate measurements was then largely determined by the rate of transfer of gas through the liquid surface in the experimental vessel and, to some extent, by the rate of mixing of the gas phase within the vessel. The time resolution of our measurements (speed of response to an instantaneous pressure change in the gas under observation) was systematically investigated for oxygen and for carbon dioxide over a range of liquid volumes in the experimental vessel, and for different inert carrier gases. Under the conditions of the measurements reported in this paper, the half-time for complete response in different experiments was between 8 and 11 seconds. This was only slightly slower than the manometric response, measured with the same vessel on an ordinary Warburg manometer shaken in exactly the same fashion. Measurements with a vessel containing only buffer established that, under the chosen conditions, half-times for equilibration of CO_2 and of O_2 were the same within experimental error.

Prior to measurement, cells were washed twice with glass-distilled water and suspended either in ca 0.05 M KH_2PO_4 solution or in fresh culture medium (pH 4.6). The experimental suspension was equilibrated in the reaction vessel with helium gas containing about 2 % O_2 and varying amounts of CO_2. During measurement, the cells were illuminated with an

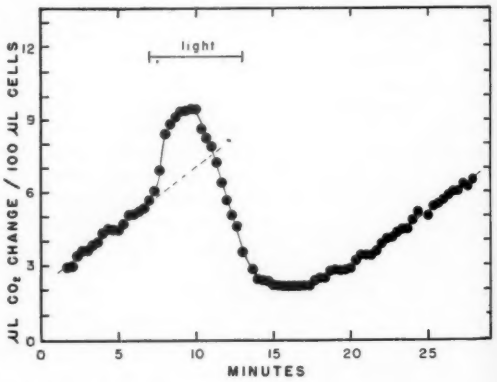
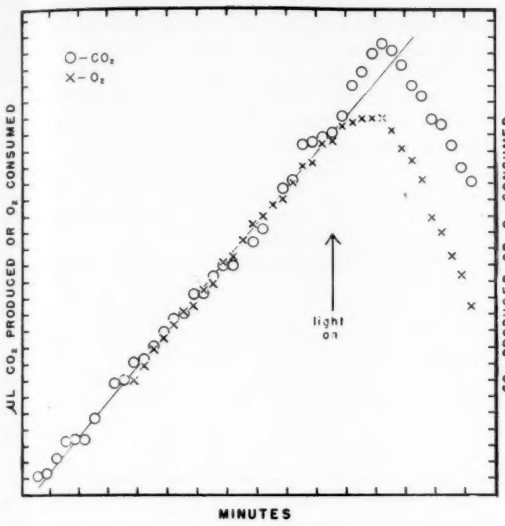


Fig. 1. Representative CO_2 burst and subsequent gulp in Chlorella. Cells grown 5 days with tungsten light at 25° C. Culture dark 1 day (25° C) and 15 hrs (5° C) with 5 % CO_2 in air bubbled through culture continuously. Experimental suspension, 290 μl cells in 3.5 ml 0.048 M KH_2PO_4. Measuring light, white fluorescent. Shaking rate, 182 oscillations per min at 17 mm vessel excursion. Bath temp, 14 5° C.



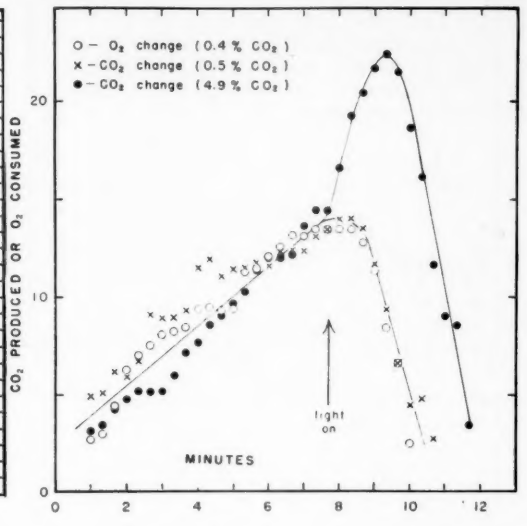


FIG. 2 (*left*). Smaller CO_2 burst by Chlorella cells grown in fluorescent light at room temperature (25 to 28° C). Culture transferred to low intensity tungsten light at 5° C 15 hrs before harvesting; 5 % CO_2 in air bubbled through culture continuously. Experimental suspension, ca 200 μl cells in 3.5 ml 0.048 M KH_2PO_4. Shaking rate 200 oscillations per min at 17 mm vessel excursion. Measuring light, white fluorescent. Gas phase, 2.0 % O_2 and 2.2 % CO_2 in He. Bath temp, 14.5° C. Data are for consecutive illumination periods superimposed on the time axis at the beginning of illumination.

FIG. 3 (*right*). Elimination of burst by reduced CO_2 pressure. Chlorella cells grown in tungsten light at 15° C and at reduced light intensity at 5° C for 20 hrs before harvesting; 5 % CO_2 in air bubbled continuously through culture during cell growth. Experimental suspension, 280 μl cells in 3.5 ml 0.048 M KH_2PO_4. Shaking rate, 200 oscillations per min at 17 mm vessel excursion. Bath temp, 15° C. Measuring light, white fluorescent. Experimental gas phase, 2 % O_2 in He with CO_2 concentrations as indicated. The 3 sets of time course data are compared by superimposing at the beginning of illumination.

intensity such that the apparent rate of photosynthesis was 2 to 4 times the rate of respiration. It was unimportant whether fluorescent or tungsten light was used. Helium was preferred as a carrier gas since it was found that the overall response time of the mass spectrometer to changes in partial pressure of CO_2 or O_2 was appreciably less in helium (or hydrogen) than in other common gases. This was not caused by differences in the rate of transfer of gas across the liquid-gas interface, but rather by different rates of mixing in the gas phase itself. For a particular experiment, the conditions of cell culture and of the measurements are described in the legend of the corresponding figure.

RESULTS

Preliminary observations showed that with Chlorella no significant pressure anomalies at the onset of illumination were attributable to masses 2, 28, or 32, thus eliminating H_2, CO, N_2, and O_2 as possible contributors to the pressure burst.

The time course of oxygen metabolism showed a brief induction period at the beginning of illumination, followed by continuous production at a steady rate. No abnormal transients were associated with either the dark-light or light-dark transitions. When CO_2 (mass 44) was determined (fig 1), a marked production was observed immediately after the light was turned on (in this instance, at a rate about 7 times the preceding dark respiratory rate). When the light was turned off, CO_2 production did not start for several minutes, the intervening period being occupied by a "gulp" or uptake of CO_2.

The possibility that the CO_2 burst was the result of heating of the suspension by illumination was eliminated by control experiments with a suspension of dead cells.

The magnitude of the "burst" was markedly dependent upon culture conditions. Large bursts frequently were favored by growing the cultures at 12 or 15° C rather than at higher temperatures. Tungsten illumination during culture yielded cells capable of larger bursts than did fluorescent illumination, although the intensities were such as to give approximately equal growth rates. Figure 2 shows data for cells cultured in fluorescent light. The rate of production of CO_2 shows only slight enhancement immediately after the light is turned on. However, comparison with the time course for O_2 production shows an apparently longer induction time for CO_2 than for O_2. This delayed consumption of CO_2 may be taken as evidence of a small CO_2 burst balancing consumption by photosynthesis. If the actual time course is assumed to be the same for oxygen production and

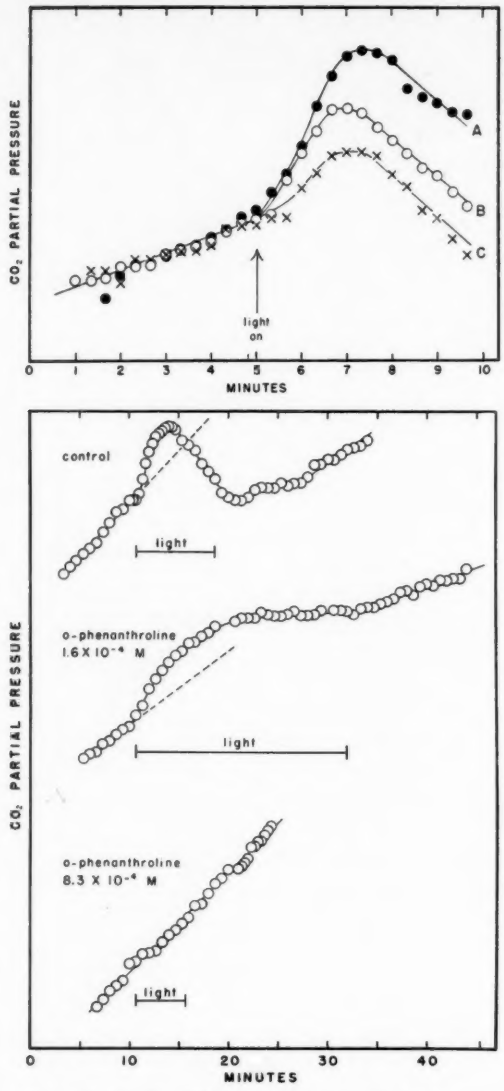


FIG. 4 (*above*). Effect of preceding dark time on magnitude of CO_2 burst in Chlorella. Cells grown 3 days with tungsten illumination (25° C), one day at reduced light intensity (25° C), 12 hrs with reduced light intensity at 12° C, then 15 hrs at same low light intensity at 5° C; 5 % CO_2 in air continuously bubbled through culture. Experimental suspension, 400 μl cells in 3.5 ml 0.048 M KH_2PO_4. Measuring light, white fluorescent. Shaking rate 182 oscillations per min at 17 mm vessel excursion. Bath temp, 14.5° C. Gas phase, 2.3 % CO_2 and 2 % O_2 in He. A. Time course of CO_2 burst following 20 min dark interval. B. Time course of CO_2 burst when illumination schedule was 10 min dark, 5 min light (average of two cycles). C. Time course of CO_2 burst when illumination schedule was 5 min dark, 5 min light (average of two cycles). Data A, B, and C are shown superimposed at the beginning of illumination in each case.

for CO_2 consumption, differences in the net induction curves for O_2 and for CO_2 may in general be interpreted in terms of a CO_2 burst.

The burst was increased with increase of CO_2 concentration up to about 5 %. Reduction in this concentration to 0.5 % reduced the burst to an insignificant value, whereupon the time course for O_2 production and that for CO_2 consumption were identical within the error of measurement (fig 3). Restoration of a higher concentration of CO_2 caused the reappearance of the burst.

The magnitude of the CO_2 burst is also determined by the length of the preceding dark time. With periods of about 20 minutes in the dark, the burst in the following light period was nearly maximal; further increase in dark time did not appreciably increase the burst. When the dark period was reduced to 10 minutes, alternated with 5 minutes light, the burst was somewhat diminished; with alternate 5 minutes light and 5 minutes dark, the burst was maintained at about 1/3 its maximal value (fig 4).

If, during the dark period, a low partial pressure of oxygen was maintained (by flushing the system with helium immediately before the light was turned off), no net production of CO_2 was observed in the following light period. Not only was the burst absent but there was a prolonged anaerobic induction phase. This might be at first sight attributed to a small burst, but comparison with the time course of oxygen metabolism shows that, under these conditions, both O_2 and CO_2 exhibit prolonged inductions. This effect was reversible; addition of O_2 during the dark restored the ability of the cells to produce a burst.

Emerson and Lewis distinguished two phases in the time course of CO_2 production during the burst, the integrated areas under the two portions of the curve being approximately equal. Our method did not resolve a second phase. If quantitative comparison is made between the first phase of Emerson and Lewis's burst and the corresponding period in our data, then the magnitude of the burst in our experiments was about half that in theirs. Furthermore, the maximal initial rate of production of CO_2 in our experiments was about 7 times the respiratory rate, compared with a rate of some 15 times in the experiments of Emerson and Lewis. This suggests that we did not achieve the culture conditions needed to maximize the burst. Nevertheless, our observations confirm essentially all the qualitative characteristics originally reported and demonstrate that, with our cultures, the burst is of such magnitude that its effect could not be disregarded in manometric measurements.

FIG. 5 (*below*). Differential effect of *o*-phenanthroline on CO_2 burst and photosynthesis. Chlorella cells grown with tungsten illumination. Last 15 hrs before harvesting cells culture was in the dark at 5° C; 5 % CO_2 in air bubbled through culture continuously. Experimental suspension, 250 μl cells in 3.5 ml 0.048 M KH_2PO_4. Bath temp, 14.5° C. Measuring light, white fluorescent.

Emerson and Lewis left some uncertainty as to the quantitative importance of the burst in light periods subsequent to the first few exposures when cells are subjected to repeated alternate light and dark periods of brief duration. Whereas they stressed the fact that 10 minutes was too short a dark interval for the succeeding burst to be maximal, our observations clearly show that even with 5-minute alternations the burst is repeated in each light period and has a far from negligible volume. Even with alternate periods of 1 minute light and 1 minute dark we observed a significant difference in the time courses of O_2 and of CO_2, which may be attributed to the effect of the burst persisting even with such short time intervals. It is thus clear that when alternate light and dark periods are used for quantum efficiency measurements, the burst may play an important role, even if the light and dark periods last only a few minutes.

Photosynthetic fixation of CO_2 and the CO_2 burst were observed to be differentially inhibited by cyanide and *o*-phenanthroline. The *o*-phenanthroline (1.6×10^{-4} M) inhibited photosynthesis almost completely before the burst was appreciably affected (fig 5). On the other hand, cyanide (2.8×10^{-3} M) completely inhibited the burst while much of the photosynthetic ability of the cells was retained (fig 6). In both cases the inhibitor was added after a dark time sufficiently long for the CO_2 "reservoir" to be almost completely filled. Thus the experiment shows that the liberation of CO_2 in the light is inhibited by cyanide.

Emerson and Lewis interpreted the burst as a rapid photochemical emptying of some sort of CO_2 "reservoir" and the post-illumination gulp, as a slower refilling of this reservoir. In keeping with that interpretation, it appears from measurements of the influence of dark time on the ability of cells to show a burst, that the reservoir refills with a half-time between 5 and 10 minutes.

By introducing tracer CO_2 into the gas phase it is in principle possible to determine whether the reservoir can exchange CO_2 with the environment. In the experiment illustrated by figure 7, cells which had demonstrated large CO_2 bursts were given a sufficiently long dark time to insure that the reservoir was practically full. $C^{13}O_2$ was added to the gas phase at zero time and its concentration continuously measured. The light was turned on 4 minutes after the addition of tracer. The figure shows that an appreciable burst—in this case $C^{13}O_2$—was observed. In a similar experiment, a significant production of $C^{13}O_2$ was detected upon illumination after only 2.5 minutes exposure to tracer. Illumination periods of about 20 and 40 minutes after the addition of tracer led to larger $C^{13}O_2$ bursts, as isotopic labelling of the reservoir approached saturation. Subsequent meas-

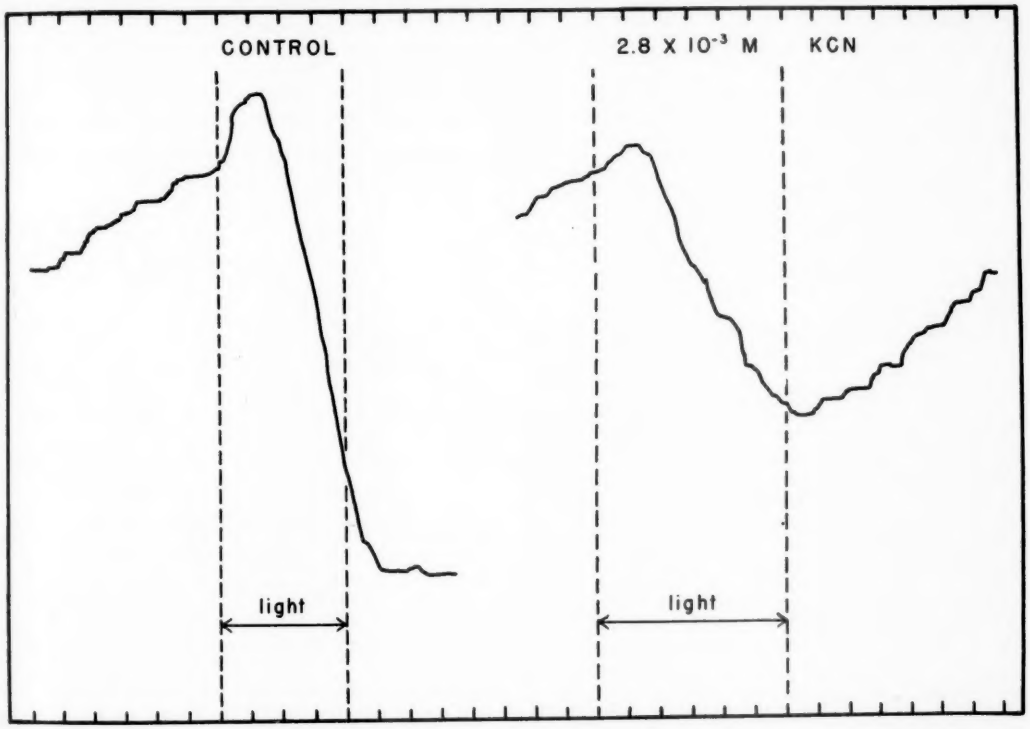


FIG. 6. Effect of cyanide on the CO_2 burst and on photosynthesis. Same cell suspension as for figure 3. Original data traced from chart of recording potentiometer. Vertical potentiometer displacement is directly proportional to CO_2 partial pressure. Each horizontal division represents one minute.

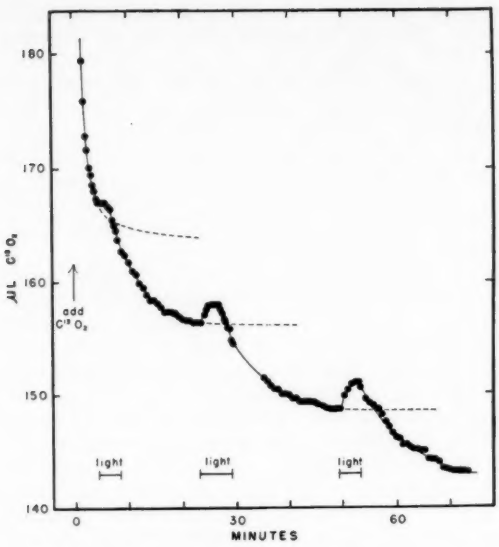


FIG. 7. Labelling the burst reservoir with tracer CO_2 and evidence for photoinfluence on isotope exchange. Cell suspension same as for figure 1.

urements of bursts of $C^{12}O_2$ demonstrated that, after many minutes, the ratio, $C^{12}O_2/C^{13}O_2$, in the burst approximated the isotope ratio in the gas phase, as would be expected if the reservoir approached equilibrium with environmental CO_2.

The initial rapidity of tracer uptake (isotope exchange) precluded reliable measurements of the burst volume immediately after adding tracer. Therefore, it was not possible to obtain an accurate estimate of the rate of just that exchange reaction with which the burst-reservoir alone is concerned. It may be concluded only that, when the reservoir is full, isotope exchange with CO_2 is as fast and, probably, faster than the refilling of the empty reservoir in the dark after a light period.

An unexpected feature of figure 7 is the enhancement of the rate of $C^{13}O_2$ uptake immediately after each illumination period. The dark rate of uptake, extrapolated from other observations of dark exchange preceding the light period, is shown by the dashed line. The pick-up of CO_2 in the dark (chiefly gas-liquid equilibration) is completed within a minute, so that the prolonged uptake of $C^{13}O_2$ observed in this experiment, at a rate much greater than the extrapolated dark rate, may be interpreted as photoacceleration of the exchange of environmental CO_2 with the carbon of cellular components present in an amount appreciably greater than the burst reservoir itself. This observation is of interest in relation to previous observations on postillumination "pick up" as determined with $C^{14}O_2$. Gaffron et al (7) reported that the incorporation of C^{14} in the dark immediately following a light period declined with a half-time of about 3 sec when CO_2 was present during illumination. Moreover, these workers found the maximal "dark pick-up" subsequent to illumination to be equivalent to approximately 10 sec photosynthetic fixation. In contrast, our results show a much greater CO_2 exchange as a result of brief illumination, and the enhanced exchange declined much more slowly than in the experiments of Gaffron et al. Chemical identification of the substances labelled was not attempted in the present work. Our observations also make dubious the assumption of Van Norman and Brown (10) that isotope exchange rates were not altered by illumination, under experimental conditions comparable with those prevailing in the present experiments.

The CO_2 burst is not confined to Chlorella. With *Scenedesmus obliquus* (Gaffron's strain D_3), grown under the same light and temperature conditions as were effective for Chlorella, significant CO_2 bursts were observed upon illumination. Figure 8 shows two time courses of CO_2 metabolism by a Scenedesmus suspension. In curve A, a small burst is evident, especially when contrasted with curve B, in which the burst is absent, due to the low concentration of CO_2 which prevailed.

SUMMARY

The observations of Emerson and Lewis on the carbon dioxide burst in *Chlorella pyrenoidosa* were substantially confirmed by an independent method. Gas exchange was measured by mass spectrometry which is specific both for CO_2 and for O_2. When cells were cultured according to the conditions reported by Emerson and Lewis to result in maximal deviations of pressure from the steady state rate at the beginning and end of illumination, prominent changes in CO_2 were observed with the present method. Identification of the gas responsible for the pressure changes

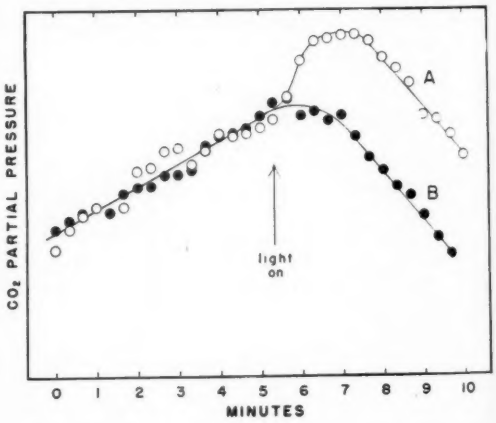


FIG. 8. CO_2 burst by Scenedesmus cells and elimination of burst by low CO_2 conc. Cells grown at 15° C in tungsten light and for the last 24 hrs at 5° C at reduced light intensity. Measuring light, white fluorescent. Shaking rate, 199 oscillations per min at 17 mm vessel excursion. Bath temp, 15° C. Curve A. Gas phase: 2.2 % CO_2 and 2 % O_2 in He. Curve B. Gas phase: ca 0.1 % CO_2 and 2 % O_2 in He. Data for separate experimental periods superimposed at the beginning of illumination.

was unequivocal; O_2, N_2, H_2, and CO were specifically eliminated as significant contributors to the pressure transients. The burst was observed to depend on both O_2 and CO_2 tensions during the preceding dark period. The magnitude of the burst also depended on the duration of the dark interval before illumination. The half-time for restoration of maximal burst capacity was about five minutes. The phenomenon was not eliminated by cyclic repetition of dark and light periods; each illumination produced a burst whose magnitude depended on the length of the preceding dark period. When cells capable of exhibiting a burst were exposed in the dark to $C^{13}O_2$, production of $C^{13}O_2$ was observed at the beginning of the subsequent illumination. Isotope exchange between environmental CO_2 and the burst reservoir was at least as rapid as the dark refilling of this reservoir. Differential inhibitions of steady state photosynthesis and of the CO_2 burst were observed with potassium cyanide and with *o*-phenanthroline.

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FAT METABOLISM IN HIGHER PLANTS. VI. INCORPORATION OF P^{32} INTO PEANUT MITOCHONDRIAL PHOSPHOLIPIDS[1]

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Phospholipids are a heterogeneous group of substances having in common the presence of fatty acids, alcoholic esters of phosphoric acid and usually a nitrogenous base among their hydrolysis products. There is considerable knowledge concerning the composition of phospholipids found in plants, but there is a complete lack of information relative to their mode of synthesis and function in the plant kingdom.

Investigations on the pathway of synthesis of phospholipids in animal tissues have been underway for some time. The tracer technique has been widely used to elucidate the mechanism of synthesis. The incorporation of P^{32} into phospholipids by animal tissues has been known since 1941 (1). Evidence has been acquired which indicates one route of biosynthesis is a phosphorylation of glycerol followed by esterification of the glycerol by long chain fatty acids to form phosphatidic acids. It has been demonstrated that isolated rat liver mitochondria incorporate P^{32} into the phospholipid fraction under conditions conducive to oxidative phosphorylation (2, 3). Addition of glycerol strongly stimulated this incorporation (3). Isotope dilution experiments and use of *a*-glycerophosphate^{32} indicate that the route of synthesis apparently proceeds by way of L-*a*-glycerophosphate (3). Esterification of *a*-glycerophosphate by long chain fatty acids requiring coenzyme A (CoA) and adenosine triphosphate (ATP) has been observed with a lyophilized guinea pig liver fraction (4). The product of this incorporation appears to be a phosphatidic acid (3, 4). Rat liver acetone powder extracts contain a system which incorporates palmitate-1-C^{14} into rat liver phosphatides (5). CoA stimulated this incorporation. Two systems have been found in rat liver which will incorporate choline into the phospholipid fraction. Kennedy (6) has isolated a system in rat liver mitochondria which in-

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corporates choline into phospholipid by a pathway not involving phosphoryl-choline. Kornberg and Pricer (7) have prepared a system from rat liver which converts C^{14} and P^{32} labeled phosphorylcholine into water-insoluble compounds soluble in lipid solvents. This paper will describe the conditions necessary for incorporation of P^{32} into the lipid phosphorus fraction of mitochondria isolated from germinated peanut cotyledon homogenates.

Materials and Methods

Mitochondria were prepared as described by Stumpf (8). Inorganic P^{32} solutions (obtained from the Donner Radiation Laboratory) were made 1 *N* to HCl and hydrolyzed for 30 minutes at 100° C to decompose any labile phosphate polymers. After hydrolysis, the solution was made alkaline to phenolphthalein, 20 μM carrier orthophosphate added, followed by an excess of barium acetate solution. The precipitate was sedimented by centrifugation, washed once with 10 % alcohol, dissolved in a small volume of HCl, and passed through a Dowex-50-H^+ column. The eluate was neutralized with KOH and used as the stock solution of inorganic P^{32}. Approximately 10^6 counts/min (cpm) were usually added to each vessel in an experiment involving P^{32}.

ATP^{32} was synthesized from adenylic acid (AMP) and inorganic P^{32} using rat liver mitochondria by the method of Kielley and Kielley (9) and isolated by the column chromatography method of Cohn and Carter (10) as modified by Kornberg and Barker (11). The elution from the Dowex-1-Cl^- column was begun with 0.01 *N* HCl + 0.1 M KCl which removed the AMP, ADP, and unutilized inorganic P^{32}. After optical density readings at 260 mμ indicated that AMP and ADP had been completely eluted, the developing solvent was changed to 0.01 *N* HCl + 0.2 M KCl to elute the ATP^{32} which was adsorbed on acid-washed unground Nuchar C 190 charcoal in order to concentrate the material. The charcoal was eluted four times with a 50 % acetone + 0.5 % ammonia solution, the four elutions were combined and the acetone evaporated under vacuum. The remaining solution was lyophilized almost to dryness, thawed, neutralized, and stored at −10° C. Approximately 10^6 cpm were usually added to each vessel involved in experiments with ATP^{32}.

The identity of the ATP^{32} synthesized was demonstrated by paper chromatographic analysis using the method of Cohn and Carter (10). The results showed that the only labeled compound in the lyophilized solution was ATP^{32}.

Extraction and Plating of Phospholipids: The method of extraction was a modification of the method used by Kennedy (3). When an experiment was concluded, the reaction was stopped by transferring the incubation mixture to a 12 ml conical centrifuge tube and adding 2 ml of 10 % trichloroacetic acid (TCA). The precipitate was centrifuged down and the supernatant discarded. The precipitate was resuspended and washed twice with 5 ml of 5 % TCA, and the supernatants discarded after each washing.

The washed precipitate was then suspended in 5 ml of absolute ethanol. After centrifuging, the alcoholic supernatant was decanted into a Maizel-Gerson vessel. The precipitate was then extracted twice with 5 ml of an 8 : 20 mixture of benzene and alcohol. After centrifugation, these extracts were added to the alcohol extract. Fifteen ml of water-saturated chloroform were added to the combined extracts. To remove any possible contaminating inorganic P^{32} which may have been extracted, the organic solvent solution was shaken up with 5 ml of 0.5 M K_2HPO_4 which was 2 M with respect to KCl. The phases were allowed to separate and the aqueous phase drawn off. This washing was repeated twice with the aqueous phase being removed each time. There was either no activity or only very slight activity in the wash solutions which indicated that removal or the dilution of the inorganic P^{32} was complete.

The organic phase was then dried in a 30-ml beaker under heat lamps. After drying, the residue was dissolved in 5 ml of water-saturated chloroform and transferred to a stainless steel planchet. The solution in the planchet was dried under a heat lamp and the activity in the phospholipid sample was counted directly in a Geiger-Müller counter.

Nitrogen Content of Enzyme: Total N determinations were made on several enzyme preparations to determine the variation from one preparation to another. Nitrogen was determined by microkjeldahl analysis. The total N value in mg N/ml based on four different preparations varied from 2.0 to 4.0 mg N/ml.

Experimental Results

Incorporation of Inorganic P^{32} into the Phosphatides of Isolated Mitochondria, *Cofactor Requirements:* Certain cofactors are necessary for in-

Table I

Cofactor Requirements for Incorporation of P^{32} into Phospholipids

Reaction mixture	Total cpm in phospholipid-P^{32}
Complete *	759
" − Mg^{++}	275
" − AMP	0
" − succinate	0
" + phosphate acceptors and cofactors **	646
Control, "boiled enzyme"	2

* The complete system contained 0.5 ml fresh washed mitochondria, 15 μM $MgCl_2$, 10 μM succinate, 3 μM AMP, 10 μM phosphate buffer pH 7.4, and 10^6 cpm of P^{32}. Final volume was 3 ml. Incubated 30 min in large test tubes at 30° C in a constant temperature room on a shaking apparatus. Air was the gas phase.

** The mixture of phosphate acceptors and cofactors contained in addition to the above components 10 μM glycerol, 10 μM inositol, 10 μM ethanolamine, 10 μM choline, 20 μM DL-serine, 3 μM L-α-glycerophosphate, 0.5 mg galactinol, 0.25 mg CoA, 0.25 mg GSH, 0.025 μM cytochrome c, 0.5 mg DPN, 0.5 mg TPN, and 1.0 mg FAD.

TABLE II

P-O RATIOS OF DIFFERENT KREBS CYCLE SUBSTRATES

SUBSTRATE	μATOMS O	μATOMS P TAKEN UP	P-O
Succinate	12.5	11.8	0.95
α-Ketoglutarate ...	4.4	2.6	0.59
Citrate	4.9	5.5	1.12

System contained 4.4 μM AMP, 1.2 μM sucrose, 15 μM $MgCl_2$, 20 μM phosphate buffer pH 7.0, 30 μM glucose, 30 μM NaF. Final volume was 3.0 ml. Into the center well of the Warburg vessel in which the material was incubated, 0.1 ml of 5 M KOH was pipetted. The vessels were equilibrated for 5 min at 30° C and oxygen readings begun. In the vessel containing succinate, the time allowed for reaction was 15 min since the oxygen uptake was so rapid that this was sufficient time to make phosphate limiting. Vessels containing other substrates were incubated to 45 to 60 min. The reaction was stopped by tipping in 0.5 ml of 35 % TCA from the side arm. The precipitate was centrifuged off and duplicate aliquots of the supernatant were used for phosphate determination by the method of Gomori (15). The phosphate uptake of the "zero time" controls was subtracted from the total P uptake and this result was used as a measure of inorganic P esterification.

corporation of P^{32} into phospholipid-P^{32}. The essential components of the reaction mixture were Mg^{++}, adenine nucleotide, and a Krebs cycle intermediate. As seen in table I, addition of phosphate acceptors such as glycerol or inositol, or other substances which probably occur as components of phospholipids failed to increase the amount of incorporation. Slight stimulations were sometimes observed when CoA and/or glutathione (GSH) were added, but the increase was rarely significant.

Omission of Mg^{++} from the reaction mixture lowered incorporation to between 30 to 65 % of that of the complete reaction mixture. A residual activity always remained even in the absence of added Mg^{++}. This activity was abolished by addition of versene (ethylenediamine-tetraacetate) to the enzyme system, but could be restored by addition of Mg^{++}, Mn^{++} can substitute completely for Mg^{++}. The adenine nucleotide requirement can be satisfied by either AMP, ADP, or ATP.

The last essential component of the incorporation system was a Krebs cycle intermediate. Succinate and α-ketoglutarate are the most effective. When citrate, malate, or pyruvate plus a catalytic amount of malate were used, addition of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) increased the amount of P^{32} fixed into phospholipid. This stimulation is in accord with results obtained on Krebs cycle oxidation by Davies (12) and Brummond and Burris (13) with plant mitochondria. Since the oxidation of a Krebs cycle intermediate is the means by which phosphate is esterified in aerobic respiration, it would appear that the Krebs cycle requirement is related to the esterification of P^{32} into ATP^{32}.

Phosphorus to Oxygen Ratio Studies: To determine the efficiency of the peanut mitochondrial system in esterifying P, the P-O ratios with different Krebs cycle substrates and the washed mitochondrial preparation were studied. The results are given in table II. With succinate, oxygen uptake was most vigorous and the total amounts of P fixed were the largest. Citrate, however, was the most efficient as a source of energy for esterification, having a large P uptake with a small mount of oxygen utilized. Since hexokinase occurs in the washed mitochondrial preparation, addition of yeast hexokinase to the succinate or α-ketoglutarate systems does not give an increase of phosphate esterification, while deletion of the glucose acceptor decreases the amount of phosphate esterified. This is in accordance with the results of Millerd et al (14), Saltman (16), and Bonner and Millerd (17).

Incorporation of P^{32} vs Time: Table III summarizes the results of a time study of P^{32} incorporation into phospholipid. As far as labeling of the phospholipid is concerned, a plateau is reached in 30 minutes. These results suggest that the pool sizes of the intermediates between inorganic P^{32} and lipid-P^{32} are quite small and thereby allow the plateau to be attained rather rapidly. It is clear from the data that only a small amount of the total P^{32} added is incorporated into the phospholipid fraction. The usual level of incorporation ranged from 0.04 to 0.35 %. However, it is also clear that this incorporation is an enzymatic process.

Inhibitory Studies of P^{32} Incorporation into Phosphatides: In order to clarify some of the intermediate steps involved in the incorporation of P^{32}, a series of inhibitors, which affect known types of reactions, were incubated with the mitochondrial reaction mixture and their effect on P^{32} fixation observed (table IV).

With succinate as the Krebs cycle substituent, malonate in substrate amounts is an excellent inhibitor of P^{32} fixation. Another effective inhibitor is dinitrophenol (DNP) which is an agent for uncoupling phosphorylation from oxidation. Just as with rat liver mitochondria (2, 3) incorporation of P^{32} into phospholipids with peanut mitochondria appears to be coupled to oxidative phosphorylation.

However, with α-ketoglutarate as the substrate, the pronounced inhibitory effect of malonate was lost. In addition, DNP had little if any effect on P^{32} incorporation. Arsenite at 5×10^{-4} M inhibited

TABLE III

TIME STUDY OF THE INCORPORATION OF P^{32} INTO PHOSPHOLIPIDS

TIME IN MIN	TOTAL CPM PHOSPHOLIPID-P^{32}
0	0
10	490
20	1220
30	1700
60	1640

Reaction system contained 0.5 ml fresh washed mitochondria, 15 μM Mg^{++}, 3 μM AMP, 10 μM succinate, and 10^6 cpm of P^{32}. Final volume was 2 ml. Incubated at 30° C in air.

TABLE IV

INHIBITION OF P^{32} FIXATION WITH KREBS CYCLE ACIDS AS SPARKERS

INHIBITOR	SUCCINATE		α-KETOGLUTARATE	
	PHOSPHOLIPID-P^{32}	INHIBITION	PHOSPHOLIPID-P^{32}	INHIBITION
	cpm	%	*cpm*	%
None	2880	..	955	..
10 μmol Malonate	60	98	755	21
10 μmol Fluoride	2900	0	...	..
0.1 μmol DNP	240	92	855	10
1 μmol Arsenite	3500	0	...	..
1 μmol Iodoacetamide	2230	23	...	..

System contained 0.5 ml fresh washed mitochondria, 10 μmol substrate, 15 μmol Mg^{++}, 3 μmol AMP, and 10^6 cpm of P^{32}. Final volume was 2 ml. Incubated 30 min at 30° C.

65 to 85 %. This presumably was due to its effect at the lipoic acid activating level of α-ketoglutaric oxidation (18). These results would indicate that the incorporation of P^{32} into organic P with α-ketoglutarate as the Krebs cycle substrate occurs prior to the succinate oxidation step (19).

Results of Attempts at Isotope Dilution with Postulated Intermediates: If known amounts of potential non-radioactive phosphate intermediates are added to a reaction mixture containing P^{32}, the amount of radioactivity incorporated into the phospholipids should be inversely proportional to the isotope dilution by the non-radioactive phosphate of the potential intermediate. Thus far it has been shown that the conditions for oxidative phosphorylation must be fulfilled prior to incorporation of P^{32} into phospholipids. The most reasonable first step would be the formation of ATP^{32} and a subsequent transfer to some suitable phosphate acceptor such as glycerol in accordance with the results obtained by Kennedy (2) and Kornberg and Pricer (4). If such a reaction scheme is operating then the addition of increasing amounts of ATP will lower the incorporation into phospholipids by diluting the newly formed ATP^{32}. Also, the addition of glycerol phosphate to the reaction mixture should decrease the amount of labeling in the phospholipid fraction. When increasing amounts of ATP were added, the amount of incorporation dropped (table V) but when L-α-glycerol phosphate was added in varying amounts no effect was seen on the amount of P^{32} incorporated. That the addition of glycerol to the reaction mixture had no effect (table I) could suggest that sufficient glycerol is already present to satisfy the acceptor requirements. Glycerol would not be limiting. However, when glycerol-1-C^{14} was added, no C^{14} labeled phospholipid could be recovered.

The dilution with ATP appears to proceed linearly until high concentrations are reached and the effect tapers off. This is similar to the findings of Dawson (20) with guinea pig brain suspensions. The primary acceptor of P^{32} is probably ADP. The complete equivalence of any of the adenine nucleotides is probably due to the functioning of a mitochondrial myokinase which is known to be present from experimental work in progress. This enzyme catalyzes reaction (*1*)

$$(1) \qquad 2ADP \rightleftarrows ATP + AMP$$

At higher concentrations of ATP the equilibrium would be shifted to the left, and the leveling off of incorporation at high ATP concentrations would be a balance between the higher concentration of P acceptor, viz ADP due to the myokinase activity, and the dilution effect of the added ATP.

The results with added glycerol phosphate show no dilution effect. It may be that the glycerol phosphate acts in some way to protect the ATP^{32} formed, perhaps by acting as a competitive inhibitor for a non-specific phosphatase which in the absence of glycerol phosphate is an effective ATPase. L-α-glycerol phosphate, a gift from Dr. E. Baer, was used since work by Baer et al (21), and Long and Maguire (22) has shown that the natural phosphoglycerides have the L-α-configuration. In addition, no effect was observed on P^{32} incorporation when inositol monophosphate, phosphorylcholine, or galactinol were added to the reaction mixture.

Identification of ATP^{32} as a Reaction Product: The identification of ATP^{32} in the reaction mixture was undertaken to demonstrate that it was a product formed by P^{32} uptake. An experiment was run in which carrier P was added in one case and no carrier in the second. The supernatants were made alkaline to phenolphthalein and 10 μM carrier P added to the supernatant which had been incubated carrier-free. The inorganic P was precipitated in both by magnesia mixture (23) and centrifuged off and discarded. Two μM of carrier ATP were added to each supernatant and precipitated down, dissolved in dilute HCl, and shaken with a small amount of Dowex-50-H^+ resin to remove the Ba^{++}. The resin was

TABLE V

EFFECT OF ADDED ATP AND THE INCORPORATION OF L-α-GLYCEROLPHOSPHATE INTO PHOSPHOLIPIDS

ATP DILUTION		GLYCEROLPHOSPHATE DILUTION		
ATP ADDED	PHOSPHOLIPID-P^{32}	GLYCEROL-PHOSPHATE ADDED	PHOSPHOLIPID-P^{32}	
			A	B
μM	*Total cpm*	*μM*	*Total cpm*	
3	3072	0	825	1510
6	1340	6	1125	....
9	1027	10	....	1824

ATP system: 0.5 ml fresh washed mitochondria, 10 μM Mg^{++}, 10 μM succinate, 0.25 mg CoA, 0.25 mg GSH, 10 μM phosphate pH 7.4, and 10^6 cpm of P^{32}. Final volume was 2 ml. Incubated at 30° C.

Glycerolphosphate system: Same as above plus 3 μM AMP.

removed by centrifugation. The clear supernatant was neutralized with KOH and then chromatographed on Whatman No. 1 filter paper along with known AMP, ADP, and ATP in the two-phase system described by Cohn and Carter (10). The known adenine nucleotides were located by their quenching under ultra-violet light while the P^{32}-labeled compounds were located by radioautography. Labeled ATP and ADP were found in both supernatants. The radioautograph of the reaction mixture to which no carrier P had been added had a very dark ADP spot and a light ATP spot. When carrier P was added, the ADP and ATP spots were of the same intensity.

Paper Chromatographic Analysis of Mitochondrial Phospholipid: After counting in a Geiger Müller counter, the phospholipid was dissolved in chloroform, washed with the $K_2HPO_4 + KCl$ solution and the organic phase concentrated under heat lamps. A sample of the chloroform solution was spotted on Whatman No. 1 filter paper for paper chromatographic analysis. Known samples of L-α-dipalmitoyl lecithin and L-α-dimyristoyl cephalin, kindly furnished by Dr. E. Baer, were chromatographed alongside. The chromatogram was run in the ascending manner in 8:1 ethanol-water (24) as the developing solution. After drying the labeled phospholipid was located by radioautography. The paper was then sprayed with the Hanes and Isherwood reagent (25) for organic phosphate to locate the known phosphoglycerides which had been added. Known lecithin had an R_f of 0.9 and cephalin streaked with an approximate R_f of 0.65. Three organic phosphate spots were detected in the chloroform extract of the plant mitochondrial phospholipid. The R_fs of these spots were (a) 0.90, (b) 0.25, and (c) 0.07. Based on spot area and color intensity, the compound running to 0.9 appeared to be the greatest in concentration. However, the radioautograph showed that labeled phospholipid was associated with the phosphate compound at R_f 0.07 and chromatographed as a very distinct spot. Phosphatidic acid, chromatographed in the same solvent system and sprayed with the Hanes-Isherwood reagent, gave a streak having a range in R_f from 0.0 to 0.2. Thus in summary the phospholipid which becomes labeled with P^{32} is neither lecithin nor cephalin, nor phosphatidic acid.

INCORPORATION OF P^{32} IN PHOSPHOLIPIDS USING ATP^{32} AS A SOURCE OF LABEL, *Cofactor Requirements of ATP-P^{32} Incorporation:* As summarized in table VI, ATP^{32} can replace P^{32} as source of label. The only cofactor necessary appears to be Mg^{++}; Mn^{++} could replace the Mg^{++} completely and Ca^{++} could replace Mg^{++} to a limited extent. A metal remains associated with the preparation since omission of Mg^{++} only lowers the incorporation by 55 %. Addition of a chelating agent such as versene completely abolished the incorporation and this inhibition can be removed by adding stoichiometric amounts of Mg^{++} or Mn^{++} and to some extent Ca^{++}.

When succinate was omitted, there was a 25 % drop in incorporation. The higher incorporation with added succinate is explained if it is assumed that a phosphatase occurs which splits P^{32} from ATP^{32}. Oxidation of succinate would re-esterify the hydrolyzed P^{32}. Thus, while omitting succinate from the inorganic P^{32} system completely abolished fixation, the omission of succinate in the ATP^{32} system has only a relatively slight effect. In support of this concept is the observation that DNP reduces the ATP^{32} system about 30 % while it inhibits the P^{32} system about 75 to 90 %.

TABLE VI

COFACTOR REQUIREMENTS FOR FIXATION OF P^{32} FROM ATP^{32}

REACTION MIXTURE	PHOSPHOLIPID-P^{32}
	Total cpm
Complete *	2020
" $-Mg^{++}$	920
" - Succinate	1515
" - (CoA + GSH)	1870
" - CoA	2090
" + 0.1 μM DNP	1330
Control **	20

* Complete system: 0.5 ml fresh washed mitochondria, 10 μM Mg^{++}, 10 μM succinate, 3 μM carrier ATP, 10^6 cpm ATP^{32}, 0.25 mg CoA, 0.25 mg GSH.

** Control: The enzyme was heated 5 min in a boiling water bath prior to addition to the reaction mixture. Final volume was 2.0 ml. Incubated 30 min at 30° C.

Stability of the ATP^{32}-utilizing System: All attempts to prepare frozen or lyophilized mitochondrial preparations which retained the ability to fix P^{32} in phospholipids were unsuccessful.

However, when frozen mitochondria were tested for their ability to employ ATP^{32} at the source of label, they were found not to have lost their ability to incorporate P^{32} from ATP^{32} into phospholipids despite in some cases having been stored for 3 months at −10° C. Lyophilized mitochondria were prepared from washed and frozen mitochondrial preparations by centrifuging down the mitochondria, resuspending in cold water, and lyophilizing. The lyophilized mitochondria, suspended in cold water for use as the enzyme course, retained their ability to use ATP^{32} as a source of labeling of phospholipids. Five percent (by weight) suspensions were found to give adequate activity. It seems apparent that the labile enzymes are those involved in the initial fixation of P^{32} into ATP^{32} while the system employing ATP^{32} appears fairly stable. Attempts at solubilizing the ATP^{32} system were uniformly unsuccessful.

Time Study of ATP^{32}-Utilization: The effect of time on the labeling of phospholipids by ATP^{32} was studied. No further incorporation was observed after 11 minutes of reaction time. With inorganic P^{32} as the label source, the incorporation was completed within 30 minutes. Apparently most of the time required with the P^{32} system is synthesis of ATP^{32}. The fact that incorporation is more rapid from ATP^{32}

is in line with a reaction scheme that ATP^{32} is an intermediate between P^{32} and phospholipid-P^{32}.

Anaerobic Formation of Labeled Phospholipid: If the only function of the Krebs cycle acid is to provide energy for the formation of ~ P, then the synthesis of labeled phospholipids from ATP^{32} should be anaerobic in nature. A comparison of the amount of incorporation into phospholipids starting from P^{32} and ATP^{32} under aerobic and anaerobic conditions was made (table VII).

While the labeling of phospholipids by inorganic P^{32} is oxygen dependent, the phospholipid is labeled under anaerobic conditions with ATP^{32} as the source of label. Addition of agents which uncouple phosphorylation from oxidation, such as arsenate or DNP, had no effect on the amount of P^{32} fixed in the phospholipid when ATP^{32} was used.

Isotope Dilution Experiments: If the path of synthesis proceeds by $P^{32} \rightarrow ATP^{32} \rightarrow \rightarrow \rightarrow$ phospholipid-P^{32}, then increasing amounts of non-radioactive ATP added to the reaction system should decrease the incorporation in the phospholipid fraction when P^{32} is the source of the label; unlabeled inorganic P should have no effect when ATP^{32} is the source of label. Tables V and VIII give the results of such an experiment. Increasing amounts of unlabeled ATP decreased the amount of activity in the phospholipid. Diluting amounts of unlabeled P had a small effect. The latter is probably due to dilution of the ATP^{32} by synthesis of "cold" ATP with the oxidation of small amounts of Krebs cycle acid present as a contaminant in the washed mitochondrial fraction.

Results with P^{32} discussed previously had shown no stimulatory effect on addition of glycerol or inositol to the reaction mixture. It seemed possible that these compounds might not have been able to penetrate the mitochondrial membrane. However, with frozen or lyophilized mitochondria, there should be no permeability considerations involved. When glycerol and/or inositol were added to reaction mixtures with frozen or lyophilized mitochondria and ATP^{32}, no increase of labeling in phospholipids was observed. Thus, there is either sufficient P-acceptor present so as not to be limiting, or these compounds are not the phosphate acceptors. When glycerol-1-C^{14} was incubated with mitochondria, the isolated phospholipid fraction had no C^{14}-activity. Further, choline-1,2-C^{14} under similar conditions was not incorporated into phospholipids.

The possibility still existed that glycerol phosphate was on the pathway to phosphatide synthesis and that the negative results with previous isotope dilution experiments were due to the impermeability of the mitochondrial membrane to L-α-glycerol phosphate. However, since washed mitochondria readily oxidize L-glycerol phosphate to triose phosphate, impermeability must be ruled out as a factor.

TABLE VII

COMPARISON OF AEROBIC AND ANAEROBIC FORMATION OF LABELED PHOSPHOLIPID

SOURCE OF LABEL	TOTAL CPM IN PHOSPHOLIPID-P^{32}	
	AEROBIC	ANAEROBIC
P^{32}	430	10
	740	46
ATP^{32}	375	330
	408	456

P^{32} system: 0.5 ml fresh washed mitochondria, 10 μM Mg^{++}, 3 μM AMP, 10 μM Krebs cycle intermediate, 10 μM phosphate buffer pH 7.4, 10^6 cpm P^{32}, 0.25 mg GSH. Final volume, 2 ml.

ATP^{32} system: 0.5 ml fresh washed mitochondria, 10 μM Mg^{++}, 3 μM carrier ATP, 10^6 cpm ATP^{32}. Final volume, 2 ml.

Anaerobic experiments were carried out by placing fresh washed mitochondria in the sidearm of the Thunberg tube and evacuating the tube. After evacuation the fresh washed mitochondria was tipped into the reaction mixture to start the reaction. Incubated at 30° C for 30 min.

TABLE VIII

EFFECT OF UNLABELED P ON LABELING OF PHOSPHOLIPID FROM ATP^{32}

CONC OF CARRIER INORGANIC P	PHOSPHOLIPID-P^{32}
μM	*Total cpm*
0	408
10	350
20	311

System contained 0.5 ml of fresh washed mitochondria, 10 μM Mg^{++}, 0.25 mg GSH, 3 μM of carrier ATP, and 0.5×10^6 cpm of ATP^{32}. Final volume, 2 ml. Incubated 30 min at 30° C.

DISCUSSION

The chemical nature of P^{32}-phospholipid synthesized by peanut mitochondria remains unknown. It does not resemble the conventional phosphatidyl choline, phosphatidyl ethanolamine, or phosphatidyl serine. It is not a phosphatidic acid. Dawson (20) using guinea pig brain dispersions, found P^{32} incorporated into phospholipid under conditions of oxidative phosphorylation. Fractionation of the isolated phospholipid of these brain dispersions showed that the P^{32} was not incorporated into phosphatidyl ethanolamine, phosphatidyl serine or spingomyelin and only very slowly into phosphatidyl choline (26). The diphosphoinositide of the brain dispersion had a high specific activity and an unknown lipid substance incorporated a large proportion of the P^{32} fixed. It is possible that P^{32}-phospholipid may be related to the complicated phospholipid described by Malkin and Poole (27) as a phosphoinositide since this phospholipid makes up 38 % of the total peanut phospholipid. It is conceivable, however, that the P^{32} is incorporated into as yet unknown or undescribed phosphatides.

The possibility still exists that the reaction being studied is an exchange type of reaction since it is undetermined as to whether there has been any net synthesis. Such an exchange would be an enzymatic

one. However, unless the P is a terminal group, the probability that all the bonds open simultaneously and allow P^{32} to exchange for unlabeled P is rather remote. The observation that a specific phospholipid becomes labeled would argue against a simple general exchange reaction into the several types of phospholipids.

The fact that incorporation of the added phosphate did not exceed 0.35 % does not necessarily imply that the synthetic mechanism involved is sluggish or of little consequence. The levels of incorporation here are of the same magnitude as those found by Kennedy (3) with rat liver mitochondria.

Summary

The incorporation of P^{32} into the phospholipid fraction of peanut cotyledon mitochondria has been described. The first step on the route of incorporation is the esterification of P^{32} into ATP^{32}. This step requires an adenine nucleotide, Mg^{++} and a Krebs cycle intermediate. The nucleotide requirement can be satisfied by either AMP, ADP or ATP. The incorporation into ATP^{32} is oxygen dependent and is sensitive to dinitrophenol. Only freshly prepared mitochondria can perform this step. The maximum amount of incorporation was 0.35 % of the added P.

The steps from ATP^{32} to lipid phosphorus are unknown. Mg^{++} is required, and either fresh, frozen or lyophilized mitochondria can perform this sequence. Dinitrophenol has no effect on this portion of the route of P^{32}.

The phospholipid which becomes labeled is unknown. Chromatographic studies have shown it is not phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, or a phosphatidic acid.

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THE DEVELOPMENT OF BEAN SEEDS (PHASEOLUS VULGARIS L.)[1,2]

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Though the cell has been accepted as a biological unit since the time of Schleiden and Schwann, it has been ignored as the physiological unit. Numerous studies have been made on the physiology of fruits and seeds (6, 10, 19) and the embryology of many species has been described (15, 16), yet only a few attempts have been made to examine the biochemical changes on a *per cell basis*. It is unfortunate that so few cellular studies have been made, since they might indicate cell similarities that are not apparent at the organ level. The physiological investigations on the growth of plant cells that came to the author's attention include cowpea roots (22), corn roots (8, 11), onion roots (31), pea roots (2, 4), and apple fruit pulp (1, 20, 23); these papers are considered in the discussion of the results.

The aim of this investigation was to describe the development of bean seeds in cellular terms and to compare the per cell values obtained with those of other plant cells. To this end, the fresh weight, dry weight, nitrogen (N) content, phosphorus (P) content, and the rate of oxygen uptake were measured and cell counts were made on maturing bean seeds.

MATERIAL AND METHODS

Black Valentine bush beans (*Phaseolus vulgaris* L.) were planted 4 inches apart in rows 18 inches apart in greenhouse beds containing 0.5 foot of soil. Plantings were made at 2-week intervals so that a complete range of seeds could be collected in each harvest. The plants were watered daily and no artificial illumination was used. At the start of flowering, some 40 days after planting the seeds, the newly open flowers were individually labeled daily. Approximately a week later, the number of marked flowers, which were now small pods, was reduced to one per plant. Pods from different plantings, 11 or more days past the open flower stage, were collected every day or two. If the seeds in a pod were uniform, that is the fresh weight of the lightest bean was at least 2/3 that of the heaviest, one or two of the seeds in the pod were selected at random for further study.

Whenever the material sufficed, N, P, and respiratory measurements were made on the same single bean seed sample. As cell counts involved the destruction of the seeds, only fresh weight and respiratory measurements could be made on beans used for this purpose. Cell counts were made on the cotyledons alone. The reason for this was that, on the one hand, it would have been difficult to perform several chemical analyses on the youngest pairs of cotyledons encountered due to their small size; nor would it have been possible to obtain valid rates of oxygen uptake due to wounding in excision. On the other hand, the author was unable to properly macerate entire seeds for cell counts. The experimental design permitted one to obtain measurements on individual seeds rather than only average values.

Fresh weights were taken on the selected seeds within an hour after the pods were picked, immediately after opening the pods. Samples weighing less than 25 mg were measured on a Roller-Smith torsion balance to 0.01 mg; samples weighing more than 25 mg were measured on a similar balance accurate to 0.2 mg.

Respiratory measurements were made in a Warburg manometer at 25° C. After preliminary experiments, with intact bean seeds ranging in fresh weight from 20 to 600 mg, had indicated a respiratory quotient of unity by both the direct and the indirect method, all subsequent measurements were made on oxygen uptake alone in a CO_2-free atmosphere. In addition to the alkali, no further fluid was put into the vessels. All calculations were made including the water content of the seeds in the volume of the vessel occupied by liquid, and ignoring the volume occupied by the dry constituents. The error in measuring the oxygen uptake of a seed for an hour or longer was less than 5 %.

Dry weights were taken after the respiratory measurements, on seeds that were not macerated. The beans were dried for 24 hrs at 65 to 70° C, and weighed on torsion balances. Later the seeds were crushed in a device consisting of a block of brass with a 10-mm diameter, flat-bottomed hole into which fitted a short rod. After crushing, each seed was individually ground to a fine powder with a mortar and pestle for analysis.

Nitrogen was determined essentially after the method of Thompson and Morrison (30). To convert all reduced nitrogen to ammonium ion it was important to continue digestion with H_2SO_4 and H_2O_2 for 0.5 hr beyond clearing. The samples were neutralized using bromthymol blue as an internal indicator (one drop of 0.04 % weight/volume per tube); the yellow to blue change was used. This procedure is more convenient than the use of litmus paper as an outside indicator which Thompson and Morrison (30) advise, and no appreciable error is introduced. The error of this method is approximately 5 %.

Phosphorus was determined by the method of King (14). Digestion with perchloric acid and H_2O_2

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was continued 10 min past clearing as shorter periods did not liberate maximal amounts of phosphate. (It is of interest to note that as the seeds matured, the percentage of phosphorus not released by digesting merely to clearing increased from about 10 to 40.) The error of this method is less than 5 %.

Cell counts were made using a modification of the method of Brown and Rickless (3). The seed coat and epicotyl were removed from a bean seed to be macerated and the cotyledons were infiltrated by means of a water pump with an 0.8 N HNO_3 solution containing 7.5 % weight/volume chromic acid; to avoid foaming a drop of caprylic alcohol was added before infiltration. The cotyledons remained in this solution from 12 to 48 hrs depending on the fresh weight of the seeds; for example, cotyledons from seeds weighing 25 mg were treated for 12 hrs, 100 mg for 30 hrs, and 200 mg or heavier for 48 hrs. Cotyledons from seeds weighing over 400 mg were very difficult to macerate and required repeated and prolonged infiltration. Afterwards, the macerated cotyledons were shaken violently in a stoppered tube for 2 min to separate the cells; the excess acid was neutralized with sodium bisulfite; a drop of 1 % fast green added to stain the cells; and water added to make a volume suitable for counting with a hemocytometer, that is about 300 to 200 cells in a volume of 1.6 mm^3. Four separate samples were counted from each pair of cotyledons; the lowest count was usually at least 2/3 as great as the highest. The results reported are based on the average of each set of four counts.

Results and Discussion

The data obtained were very variable. This heterogeneity is largely a consequence of a lack of a physiological age index; such indexes have been reported for vegetative Xanthium plants (9) and lily flower buds (7) but not for bean seeds. During the first 10 days past flowering, while the pods were elongating, the weight of the individual seeds could be estimated from the length of the pods. Later the respiratory activity and the percentage of water in the seeds were correlated. But for neither period was a strict relationship found, and all attempts to find even a crude age index for the entire period studied failed. Therefore the data have been presented directly against time.

The data on the dry weight, nitrogen content, phosphorus content, fresh weight, and the rate of oxygen uptake of individual seeds at different stages of development were obtained in the Spring of 1954 and are presented in figures 1 to 5. The points in these and other graphs present mean values; the vertical lines their 95 % confidence intervals, calculated using Student's t test (32). By a numerical procedure (18), the smooth trend lines were obtained. It must be remembered that the smoothed curves were derived from single measurements on many individual seeds. Such data can yield distribution curves at different stages, but do not suffice for growth curves. Quick changes that may occur in the growth process of an individual will appear to be gradual when based on smooth averages (26). As great variability was found at all stages of seed development, no great weight should be attached to the exact numerical values obtained. Remembering the limitations of the data, one can nevertheless obtain some insight into the processes occurring during bean seed development.

During the first 3 weeks past flowering, the dry weight (fig 1), N (fig 2), and P (fig 3) per seed increased slowly. At the end of this period their respective values were only about 1/6 of those finally reached. In the following 2 weeks they increased greatly. Twenty-four days after flowering, they achieved their maximum rates: 22 mg dry wt/day, 0.75 mg N/day, and 0.08 mg P/day. Older seeds continued to grow at a steadily decreasing rate; till at maturity, approximately 6 weeks after flowering, they measured 353 mg dry weight, 12.5 mg N, and 1.4 mg P. While the dry weight, N, and P of the developing seeds increased together, the percentage composition did change (table I). Twelve days after flowering, the seeds contained 6.5 % N and 1.1 % P on a dry weight basis; 20 days later, the figures had fallen to half these values. The fresh weight per seed (fig 4) paralleled the other growth parameters initially; 24 days after flowering it also reached its maximum rate of increase, 65 mg/day. While the rate then declined rapidly, the fresh weight continued to gain till 36 days after flowering a maximum of 714 mg was reached. In older seeds the fresh weight itself decreased as the seeds dried. As the beans matured, the ratio of dry wt/fresh wt per bean increased—at first steadily, but with greater maturity of the seeds more rapidly. The data indicated that growth of the seeds ceased concomitantly with their loss in fresh weight (fig 7).

Table I

Composition of Maturing Bean Seeds

Days after flowering	Percentages		
	N/dry wt	P/dry wt	Dry wt/fresh wt
12	6.5	1.1	15
22	4.5	0.7	24
32	3.5	0.4	40
42	3.5	0.4	59

The rate of oxygen uptake per seed (fig 5) was correlated with the rate of dry weight, N, and P increase, but not with their absolute values. Two weeks after flowering the rate of oxygen uptake was only 30 mm^3/hr, a week later it was 170 mm^3/hr, and 26 days after flowering a maximum of 235 mm^3/hr was reached. Seeds older than 26 days past flowering respired progressively less, till in the mature seeds no oxygen uptake could be found. Such low respiratory rates as have been reported in the literature for mature seeds, 0.37 to 0.007 mm^3 O_2 hr^{-1} gm dry wt^{-1}

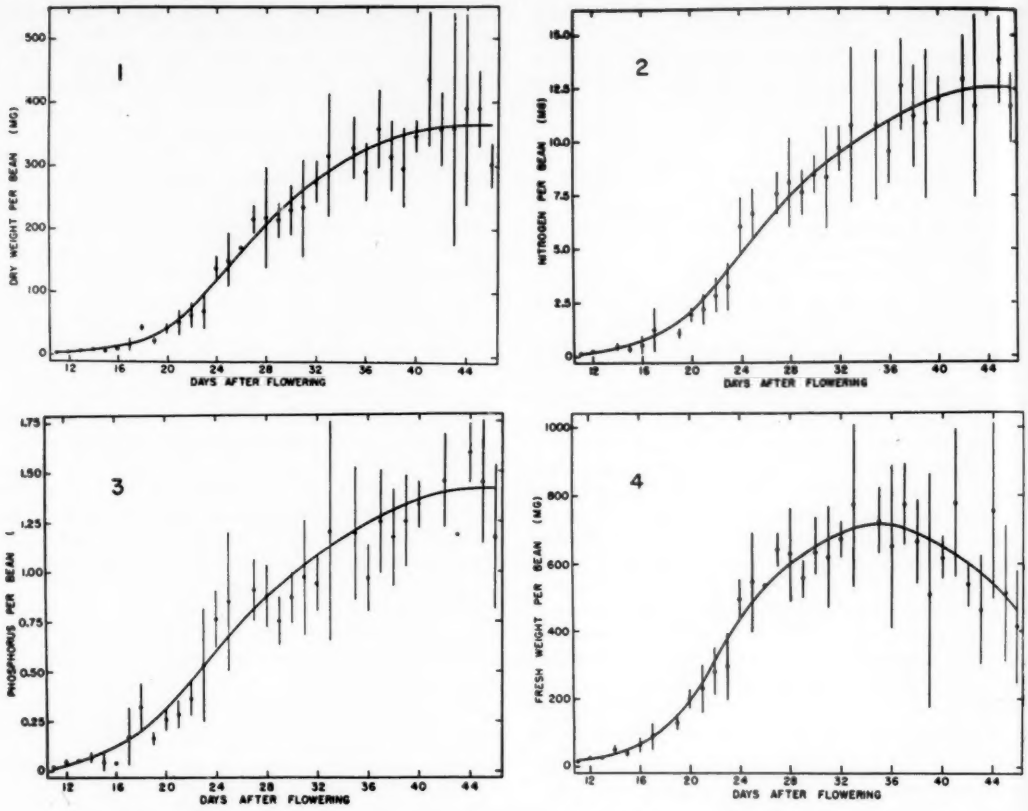


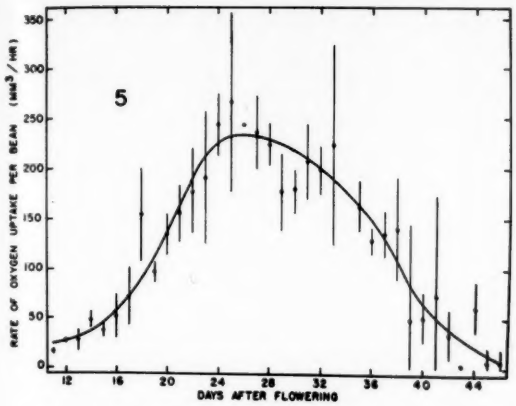
FIG. 1. Time course of the dry wt per bean seed. Curve based on 272 seeds.
FIG. 2. Time course of the nitrogen content per bean seed. Curve based on 238 seeds.
FIG. 3. Time course of the phosphorus content per bean seed. Curve based on 225 seeds.
FIG. 4. Time course of the fresh wt per bean seed. Curve based on 275 seeds.

(17), would not have been detected in the present study.

A plot of the oxygen uptake per mg dry weight against the percentage of dry matter in the seeds (fig 6) presents a curve similar to those reported by previous workers (24, 25). The highest respiratory rates were found for the youngest material studied; increasingly lower rates were found for older seeds; there were no indications of a climacteric rise. The form of this curve resembles those for the relative rates of increase, $dX/(X \cdot dT)$, in terms of dry weight, N, and P.

The cell count data, obtained in the Fall of 1953 and the Spring of 1954, are presented in figure 8. Though the seeds matured at different rates in the two periods, the counts from both groups were comparable when the fresh weights of the seeds were used for an age axis. On this account and in order to include data from seeds of unknown age, the trend curve in figure 8 was derived by smoothing the mean number of cotyledon cells of seeds having smiliar fresh weights; a secondary axis indicates the approximate age. The number of cotyledon cells per seed increased rapidly from 360,000 twelve days after flowering to 2,400,000 twenty days after flowering. Few cell divisions occurred in cotyledons older than three weeks after flowering; at this time the dry weight of the bean seeds was less than 1/6 of that finally reached. In apples (1, 20), cucurbits (27, 28), and tomatoes (12) cell division also ceases long before the growth of the fruit stops.

Though in the current study an increase in the cotyledon cells per seed from 360,000 to 2,600,000 was observed, this represents less than 3 cell generations (fig 9). In peas (5) fertilization occurs 12 to 24 hrs after the flower opens and in Lima beans (29) a day or two before the flower opens. If one assumes that the bean ovule is fertilized on the day that the flower opens and that all embryo cells divide at an equal rate, then it follows that since 18.5 cell generations have occurred by the 12th day after flowering, the average rate of cell division was a mitotic cycle every 15.5 hrs. Such a value is similar to those reported for pea root cells (2) and corn root cells (8), namely 15



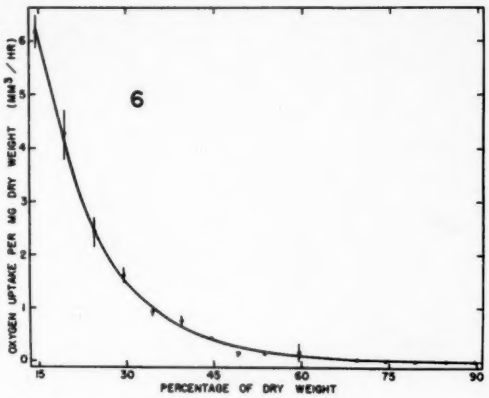


FIG. 5. Time course of the rate of oxygen uptake per bean seed. Curve based on 274 seeds.

FIG. 6. Relation between the rate of oxygen uptake per mg dry wt and the percentage of dry wt per seed. Curve based on 264 seeds.

and 14 hrs. (The latter value was obtained as the average for the distal 2 mm of the corn root; 16,000 cells are produced per hour by 223,000 cells.)

The percentage of a seed's dry weight due to its cotyledons is shown in figure 10. At nearly all stages of seed development the embryo consisted almost entirely of cotyledons; the epicotyl was never more than 2 % of the embryo's dry weight. The cotyledons grew much faster than the seed coats. In the smallest seeds studied, having a dry weight slightly over 2 mg, the seed coats accounted for 95 % of the dry weight of the entire seeds; but in mature seeds the seed coats accounted for only 10 %. The relation of the dry weight of the cotyledons to that of the seed coat can be expressed by the allometric formula (13): $y = bx^k$, where y is the dry weight of the seed coat and x, the dry weight of the two cotyledons of a seed; inspection of the appropriate plot indicated b to have a value of 0.55 and k, of 0.38. Despite the criticisms that can be made of such a formula (21), it nevertheless expresses the data concisely.

An approximation of the dry weight, N content, P content, fresh weight, and rate of oxygen uptake of the average cotyledon cell was made from the following information: (a) the values for the average bean seed at various stages of development, (b) the proportion of a seed's dry weight due to the cotyledons, and (c) the number of cotyledon cells at a given stage of seed development. An assumption made was that the seeds were uniform; i.e., on a dry weight basis the contents and the respiratory rates of the seed coat and embryo of a given bean were the same. This assumption is crucial for the youngest seeds studied since these consist predominantly of seed coat. As the epicotyl never represented more than 2 % of a seed's dry weight, its chemical composition and rate of oxygen uptake probably did not greatly influence the per seed values. It appears likely that in an immature seed, the composition and respiratory activity of the cotyledons and the seed coat do not differ much since both are composed of embryonic cells. In increasingly mature seeds, differences may well exist and increase: but as the seeds mature, the seed coats, constituting a decreasing fraction of the beans (fig 10), influence the per bean seed values less and less. Conversely, the cotyledons represent an increasing fraction of the seeds as these mature, and the composition and the respiratory activity

TABLE II

RANGE OF VALUES FOR THE AVERAGE CELL DURING DEVELOPMENT. THE SMALLER OF A PAIR OF FIGURES REFERS TO AN EMBRYONIC CELL; THE LARGER, TO A MORE MATURE ONE

CELL ORIGIN	DRY WT	FRESH WT	N	P	O_2/HR
	10^{-6} mg	10^{-6} mg	10^{-6} mg	10^{-6} mg	10^{-6} mm³
Bean seed	2–123	11–250	0.08–4.4	0.01–0.50	12–78
Apple fruit (1, 20, 23)	5–1300	30–6000	0.2 –3.6	.....	7–90
Cowpea root (22)	0.7–5	3–90	0.05–0.30	0.01–0.04	.....
Corn root (8, 11)	1–8	8–125	0.15–0.36	.....	9–48
Pea root (4)	0.5–7	10–170	0.02–0.34 *	.....	10–60
Onion root (31)	.....	.....	0.05–0.30 **	.....	6–50

* Protein nitrogen.

** Values recalculated from figure 5 in Wanner's paper (31).

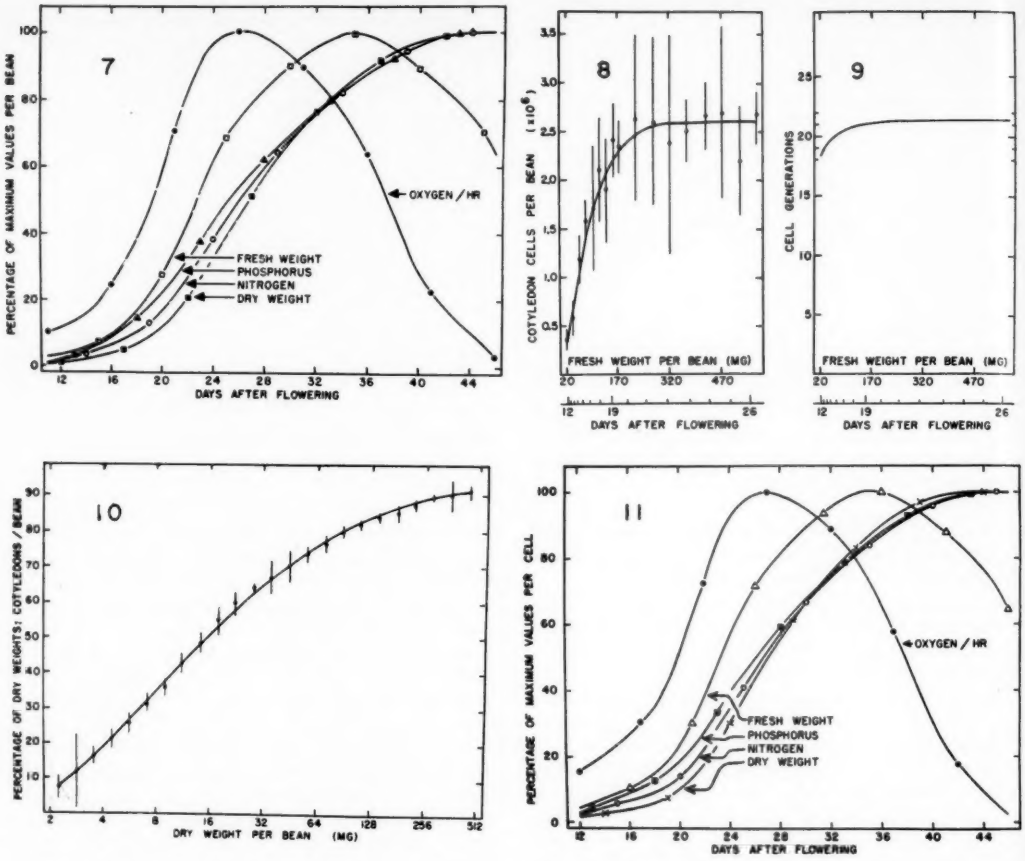


FIG. 7. Time course of the dry wt, fresh wt, N, P, and rate of oxygen uptake per bean seed. Maximum values, set at 100: dry wt 353 mg, fresh wt 714 mg, N 12.5 mg, P 1.4 mg, oxygen uptake 235 mm^3/hr.

FIG. 8. Relation between the fresh wt and the number of cotyledon cells per bean seed. Curve based on 97 seeds. Secondary age axis is approximate.

FIG. 9. Relation between the fresh wt per bean seed and the number of cell generations that have occurred since fertilization. Secondary age axis is approximate.

FIG. 10. Relation between the dry wt per bean seed and the ratio of the dry wt of the cotyledons to that of the entire seed. Curve based on 353 seeds.

FIG. 11. Time course of the dry wt, fresh wt, N, P, and rate of oxygen uptake of the average cotyledon cell. Maximum values, set at 100: dry wt 123×10^{-6} mg, fresh wt 240×10^{-6} mg, N 4.4×10^{-6} mg, P 0.5×10^{-6} mg, oxygen uptake 78×10^{-6} mm^3/hr.

of the cotyledons increasingly determine the per seed values. Therefore the assumption that the contents and respiratory activity of the seed coat and the cotyledons in a given bean were the same became increasingly unnecessary as the seeds matured.

It must be stressed that the average cotyledon cell is merely a concept: the size of the cotyledon cells in a given bean seed varied greatly, especially in the more mature stages. Thus the values obtained are at best approximations.

The shapes of the growth curves for the average cotyledon cell (fig 11) are much like those for the average seed (fig 7). The dry weight, N content, P content, and fresh weight increased gradually during the first 3 weeks after flowering. Twelve days after flowering the average cell measured 1.8×10^{-6} mg dry weight, 0.08×10^{-6} mg N, 0.013×10^{-6} mg P, and 11×10^{-6} mg fresh wt. After cell division had ceased in the cotyledons about 3 weeks after flowering, the cells grew rapidly, achieving their maximal growth rates 24 days after flowering. The rates then decreased. About 36 days after flowering the cell fresh weight started to decline, and only small dry weight, N, and P gains were made later. At maturity the

average cotyledon cell had a dry weight of 123×10^{-6} mg, and contained 4.4×10^{-6} mg N and 0.5×10^{-6} mg P.

Respiration on a per cell basis increased approximately as the rate of growth increased. Twelve days after flowering the rate of oxygen uptake of the average cotyledon cell was 12×10^{-6} mm^3/hr; 8 days later it was 40×10^{-6} mm^3/hr; and 26 days after flowering a maximum rate of 78×10^{-6} mm^3/hr was reached. On a dry weight basis (fig 6), though not on a per cell basis (fig 11), young dividing cells were found to have a more rapid oxygen uptake than more mature, rapidly enlarging cells. This correlates with the fact that even though the greatest growth was made after cell division had ceased, the relative growth rate, $dX/(X \cdot dT)$, was greatest for the youngest material studied and decreased with advancing age.

A comparison of the per cell values of bean seeds with those of apple fruits, cowpea roots, pea roots, corn roots, and onion roots (table 2) shows that meristematic cells of various origins are much more alike than more mature cells. While an average apple cell weighs 200 times as much as a mature root cell and 10 times as much as a mature bean cotyledon cell, at the youngest stages studied less than a factor of 10 separates their dry weights. A parallel situation exists in terms of nitrogen. By contrast, the rates of oxygen uptake of growing plant cells were all similar. (It should be noted that a root cell passes from the meristematic state to maturity within a day; a bean cotyledon cell requires more than a month; and an apple fruit cell, several months. The growth rates of root cells, both relative and absolute, were much greater than those of the apple and bean, even though the latter acquired the greater final size. Sinnott, studying the growth of cucurbit fruits, concluded that final size is not so much related to the rate of growth as it is to the duration of growth (27, 28).)

Despite their differences, cells of various origins have their similarities: the maximum rate of cell division and the rate of oxygen uptake per cell are comparable. It remains for further studies to determine whether or not this generality holds for other growing plant cells.

Summary

The development of bean seeds has been described on a cellular and organ basis in terms of dry weight, nitrogen content, phosphorus content, fresh weight, and rate of oxygen uptake. A comparison of the per cell values of various plants indicates that while the cells differ in size and content, their rates of oxygen uptake are comparable.

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INFLUENCE OF NIGHT TEMPERATURE AND NITROGEN NUTRITION ON THE GROWTH, SUCROSE ACCUMULATION AND LEAF MINERALS OF SUGAR BEET PLANTS[1]

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The storage roots of sugar beet plants grown in controlled climate facilities have been found to be lower in sucrose content than those normally grown under field conditions (10). When sugar beet plants were grown at 23° C under fluorescent light with an intensity of 840 fc and photoperiods of 8 hours, the storage roots contained only 3.3 % sucrose and weighed less than one-twentieth of those grown in sunlight for the same time period and air temperature. By increasing the day length in sunlight from 8 hours to a normal day of 12 to 14 hours the sucrose content of beet roots increased from 8.2 % to an average value of 9.3 %. The storage roots of the plants in the longer days doubled in size while the tops increased in size slightly. For plants in 8 hours of sunlight, decreasing night temperature from 30 to 2° C increased the sucrose content of the beet roots from 7.2 to 11.9 %, while decreasing day temperatures from 26 to 23° C increased the sucrose concentration only slightly (10). This overall failure of beet roots to accumulate sucrose at the expense of top and storage root growth is not a matter of age, since plants kept continuously in a greenhouse for natural day lengths with days at 23° C and nights at 17° C and with ample supplies of water and nutrients reached a maximum sucrose concentration of only 8.0 to 9.3 % early in the growth cycle of the plant (11). Thereafter the sucrose concentrations remained within this range until fall and winter when the values decreased to 3.9 %. The low sucrose values in winter were attributable to the short days and low light intensities which prevail during this season (11).

Whereas changes in climatic factors and in age of the beet plant have failed to produce beets above 12 % in sucrose, deficiencies in nitrogen have increased the sucrose concentrations of beets to values as high as 14 to 16 % (6). These increases, while spectacular, are still below the 16 to 20 % frequently observed in commercial fields. As a means of getting beets as high as those found in the field, a logical step to take experimentally is to study the combined effects of night temperature and nitrogen deficiency on the sucrose concentration and the quantity of sucrose stored within the root. These effects, including those upon growth and the leaf mineral content of the plant, are reported in the present paper.

Procedure

Beta vulgaris seed of the variety GW 304, treated with the fungicide Phygon XL at the rate of 1 %, was planted in vermiculite no. 2 grade on February 21, 1952, at a depth of 1.9 cm, in pots 25 cm in diameter and 30 cm deep. Each pot was provided with 4 holes at the bottom, which permitted the drainage of modified Hoagland's solution (3) added in excess daily. The seedlings from the seed balls, planted 10 to a pot, emerged on February 26, 1952. These were thinned at the 2-leaf stage to one plant per seed ball on March 12. On March 21, at the 5-leaf stage the plants were thinned to 4 plants per pot and finally, on March 28 at the 7- to 8-leaf stage, to 2 plants per pot. During this time and until August 12, 1952, the pots, 4 to a truck (51 cm × 51 cm), were kept for natural day lengths in a greenhouse maintained at 23° C from 8 A.M. to 4 P.M. and at 17° C from 4 P.M. to 8 A.M. Each week the trucks were rerandomized in order to reduce to a minimum the positional variability of the plants. Also, at weekly

[1] Received November 29, 1954.

intervals, the old leaves were removed, counted, dried, and saved for inclusion in the total dry weight of the tops of each plant.

On June 18, 1952 the pots were separated into two groups. The vermiculite in the pots of the low-nitrogen series was washed nearly free of nitrate with tap water, and thereafter these beets were watered daily with a culture solution lacking nitrogen. This solution was the same as the complete solution, except that it contained $CaCl_2$ instead of $Ca(NO_3)_2$, and K_2SO_4 instead of KNO_3.

The maintenance of specific air temperatures in the greenhouses of the Earhart Laboratory has been described by F. W. Went (12). All plants, from February 21 to August 12, 1952, were grown for natural daylengths in the greenhouse at a temperature of 23° C from 8 A.M. to 4 P.M. and at 17° C from 4 P.M. to 8 A.M. Daylength on February 21 was 11.2 hours, on June 21, 14.4 hours, and on August 12, 13.5 hours. The light intensity at noon in the greenhouse when measured normal to the sun by a Weston meter fluctuated from 5,000 to 10,000 fc. After August 12, 1952, all plants were maintained at the described day temperature, but at night (4 P.M. to 8 A.M.) they were kept at the new "night" temperatures given in figure 1. During the "night" period the plants were in fluorescent light at an intensity of 600 fc from 4 P.M. to midnight and in darkness from midnight to 8 A.M. Temperature readings of the outgoing air made at 8-minute intervals with an electronic recorder showed only minor fluctuations, and on the average were close to the assigned temperatures.

The plants from 8 pots, taken from two trucks selected at random, were processed at each harvest period. The plant material was separated as follows: (a) blades of recently matured leaves; (b) remaining living blades more than one inch wide; (c) petioles of recently matured leaves; (d) unclassified material (petioles, immature leaves, and tip of the crown); and (e) beet storage roots. The tops were separated from the roots by cutting the plants at the junction of the green and old leaves. After this separation, the beet roots were trimmed very lightly, regardless of size, by tapered trimming, as recommended for large beets in commercial tare laboratories. This procedure tended to include more crown material of the small beets than commercially practiced, but had the advantage of placing the beets of all sizes on the same basis. Pulp samples weighing 26.0 gm each were sealed separately in glass jars and frozen immediately by means of dry ice. These were analyzed for sucrose content using a saccharimeter and the hot extraction method as outlined by Browne and Zerban (1). All material from the tops of the plants was dried at 70° C in a well vented oven. After drying, the petioles and blades were ground in a Wiley mill so as to pass a 40-mesh sieve. Analyses for nitrate-nitrogen were made by the phenoldisulfonic acid method (4); phosphate-phosphorus by the phosphomolybdate method (8). Potassium was determined with a Perkin-Elmer flame photometer.

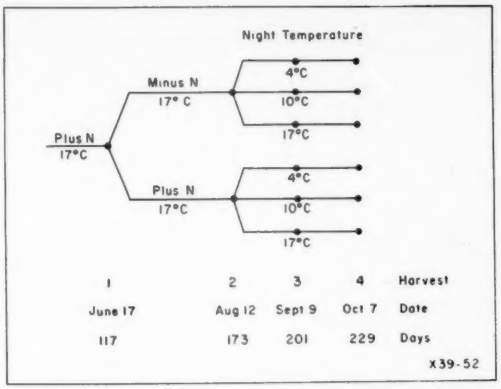


FIG. 1. Schematic diagram of nitrogen levels, night temperatures, and harvest dates for sugar beets of the variety GW 304 planted in vermiculite February 21, 1952. All plants were grown for natural daylengths in the greenhouse at a temperature of 23° C from 8 A.M. to 4 P.M. and 17° C from 4 P.M. to 8 A.M. until August 12, 1952.

All plants were watered daily with a complete nutrient solution until June 17, 1952. Thereafter one half of the plants were watered with a "complete" solution lacking nitrogen and the other half were watered as before with the complete solution, including nitrogen.

Starting August 12, 1952, all plants were kept in the greenhouse at 23° C from 8 A.M. to 4 P.M. and at the various "night" temperatures from 4 P.M. to 8 A.M. During the "night" period the plants were illuminated by fluorescent light at an intensity of 600 fc from 4 P.M. to midnight and were in darkness from midnight to 8 A.M.

The results of the experiments were analyzed statistically by the methods of analysis of variance. Error *a*, the variation between trucks within a treatment, and error *b*, the variation between pots within a truck were calculated. Error *b* was used as follows: testing treatment significance by the F-test; estimating the differences necessary for significance between treatment means (Student's t-test); and calculating the coefficients of variability (2, 5).

RESULTS

The beet root weights, sucrose concentrations, amounts of sucrose stored, top weights, leaf counts and nutrient contents of the petioles and blades are given for each harvest in tables I and II and in figures 2 to 5. A summary of the analysis of variance of each measurement is presented in tables III and IV.

SUCROSE CONTENT: The average sucrose concentration of the beet roots for the plants harvested on June 17, 1952 was 8.8 % (table I and fig 2). These plants had been grown in the greenhouse with ample quantities of water and nutrients for natural daylengths at a temperature of 23° C from 8 A.M. to 4 P.M. and 17° C from 4 P.M. to 8 A.M. As long as the plants were kept at these conditions the sucrose

TABLE I

EFFECTS OF AGE, NITROGEN NUTRITION AND NIGHT TEMPERATURE ON BEET ROOTS, FOLIAGE AND HEIGHT OF SUGAR BEET PLANTS

HARVEST DATES 1952	N LEVEL	NIGHT TEMP °C	BEET ROOT FRESH WT GM	BEET ROOT SUCROSE %	BEET ROOT SUCROSE GM	TOPS FRESH WT GM	TOPS DRY WT GM	OLD LEAVES DRY WT GM	LIVING BLADES FRESH WT GM	LIVING BLADES DRY WT GM	BLADE TO TOP RATIO ×100	HEIGHT CM
June 17	+N	17	315	8.8	28	671	54.5	34	208	21.9	30.8	57.8
August 12	−N	"	554	14.8	83	320	35.0	62	115	13.2	36.3	38.3
"	+N	"	772	9.2	72	629	57.5	70	191	21.5	30.7	47.8
September 9	−N	4	689	16.6	114	154	20.9	90	63	9.0	41.7	28.9
"	"	10	781	16.3	125	156	19.0	97	63	8.2	40.5	28.8
"	"	17	675	15.4	105	167	20.8	89	67	8.8	40.0	28.5
"	+N	4	766	11.3	86	420	45.3	93	143	19.4	34.1	41.5
"	"	10	958	10.5	100	489	49.5	107	160	20.1	32.6	46.0
"	"	17	1125	8.3	95	554	47.1	115	163	17.8	30.0	49.7
October 7	−N	4	642	18.3	118	121	17.8	93	57	8.2	47.3	21.4
"	"	10	739	18.3	135	98	14.8	96	45	7.0	46.7	19.6
"	"	17	871	15.4	134	104	13.9	100	50	6.8	49.0	21.5
"	+N	4	869	12.1	107	320	38.9	116	145	19.3	45.3	35.9
"	"	10	949	11.0	103	429	45.3	126	180	21.6	42.1	35.9
"	"	17	1113	8.8	97	489	44.4	132	156	18.3	31.8	45.8
L.S.D. (Student)		5 % level	290	1.0	36	126	14.0	26	47	6.0	4.4	5.4
" "		1 % level	385	1.3	48	167	18.6	34	63	7.9	5.8	7.1
Coefficient of variability (using error *b*)			37.6	7.7	37.0	36.7	40.1	27.5	38.9	40.5	11.3	14.9

values changed only slightly; from the 8.8 % value of June 17, to 9.2 % on August 12, 8.3 % on September 9 and 8.8 % on October 7, 1952 (fig 2). These observations are similar to those reported recently (11) in which "ripening" or "sugaring up" of beet roots is not a function of the age of the plant, but is related to environmental changes such as nitrogen deficiency or to a lowering of night temperature. On

TABLE II

EFFECTS OF AGE, NITROGEN NUTRITION AND NIGHT TEMPERATURE ON LEAF COUNT AND ON THE NITRATE-NITROGEN, PHOSPHATE-PHOSPHORUS, AND POTASSIUM CONTENT OF PETIOLES AND BLADES OF SUGAR BEET PLANTS

HARVEST DATES 1952	N LEVEL	NIGHT TEMP °C	PETIOLE NO_3-N PPM	BLADE NO_3-N PPM	PETIOLE PO_4-P PPM	BLADE PO_4-P PPM	PETIOLE K %	BLADE K %	PETIOLE DRY MATTER %	BLADE DRY MATTER %	LEAF COUNT LIVING BLADES	LEAF COUNT TOTAL
June 17	+N	17	23,700	5,930	3,340	3,140	8.70	5.04	6.8	10.5	54.6	92
August 12	−N	"	610	163	3,140	7,570	11.72	8.72	10.4	11.5	59.5	151
"	+N	"	22,100	6,650	3,750	3,060	8.97	5.52	7.9	11.1	70.1	159
September 9	−N	4	106	149	3,510	11,500	7.02	7.04	13.8	14.2	45.9	151
"	"	10	280	....	3,780	10,100	8.26	7.77	11.9	13.4	38.3	143
"	"	17	128	118	4,600	11,300	7.71	7.39	12.7	13.2	53.8	170
"	+N	4	15,600	4,670	3,930	3,380	6.59	5.20	10.3	13.4	59.6	184
"	"	10	16,600	....	3,470	2,420	7.00	4.69	9.5	12.6	67.0	200
"	"	17	19,400	4,880	4,930	3,870	7.58	4.31	7.8	10.9	54.3	180
October 7	−N	4	159	125	3,410	16,300	7.49	7.43	14.9	14.2	49.4	166
"	"	10	118	56	4,180	18,200	8.65	8.61	15.4	15.6	56.8	191
"	"	17	168	75	4,660	12,300	9.43	8.15	12.7	13.7	56.0	182
"	+N	4	16,400	5,970	3,670	3,620	7.26	6.15	10.9	13.2	52.9	195
"	"	10	18,300	6,660	3,750	3,050	7.55	6.10	9.9	12.2	67.8	225
"	"	17	23,100	4,430	4,110	3,310	9.53	4.50	7.9	11.8	60.8	229
L.S.D. (Student)		5 % level	*	**	920	3,390	1.02	0.69	1.1	1.1	10.5	21
" "		1 % level	*	**	1,210	4,500	1.35	0.91	1.4	1.5	13.9	28
Coefficient of variability (using error *b*)			.....	....	23.5	44.0	12.3	10.7	9.8	8.9	18.4	11.9

* L.S.D. (Student) − N petioles, 5 % level 177, 1 % level 237; + N petioles, 5 % level 3200, 1 % level 4300.
** L.S.D. (Student) − N blades, 5 % level 34, 1 % level 46; + N blades, 5 % level 1550, 1 % level 2080.

TABLE III

ANALYSIS OF VARIANCE OF THE EFFECTS OF TREATMENTS ON SUGAR BEET PLANTS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES									
		BEET ROOT			TOPS		OLD LEAVES DRY WT GM	LIVING BLADES		BLADE TO TOP RATIO	HEIGHT CM
		FRESH WT GM	SUCROSE %	SUCROSE GM	FRESH WT GM	DRY WT GM		FRESH WT GM	DRY WT GM		
Treatments *	12	388,027	108.565	6263.06	335,682	1,935	5,836	27,398	297.44	389.58	1152.27
Error *a* **	13	23,692	0.916	308.90	4,956	74	232	1,009	13.49	23.10	18.72
Error *b* †	78	85,028 ‡	0.988	1314.39 ‡	15,947 ‡	198 ††	659 ††	2,246 ††	36.09 ††	19.32	29.12

* All treatment effects relative to error *b* are significant at the 0.1 % level, except for living leaf count, which is significant at the 1 % level.
** Error *a* is the variance between trucks of the same treatment.
† Error *b* is the variance between pots within a truck. Error *b* significance is relative to error *a*.
†† Significance at the 5 % level.
‡ Significance at the 1 % level.

August 12, 1952 the sucrose concentration of the plus nitrogen beets was 9.2 % and of the nitrogen deficient beet roots, 14.8 %. The increase to 14.8 %, while highly significant, was still short of the 16 to 20 % reported frequently for field grown beets.

On September 9 the high nitrogen beets at night temperatures of 4 and 17° C averaged 11.3 and 8.3 % in sucrose, respectively. On October 7 the values were 12.1 and 8.8 %, respectively. For the nitrogen deficient beets the corresponding sucrose values were 16.6 and 15.4 % for September 9, and 18.3 and 15.4 % for October 7, 1952. Individual beet roots from plants deficient in nitrogen had values in October as high as 20.0 % at 4° C. Thus, by growing beets deficient in nitrogen in a climate with a low night temperature, 4 to 10° C, the sucrose concentrations obtained are similar to those of beets grown commercially. The sucrose concentrations of the beets high in nitrogen were less at 10° C than those at 4° C, whereas for the beets deficient in nitrogen, the sucrose concentrations were the same at both temperatures.

BEET ROOT WEIGHT: The average beet root weight of the plants high in nitrogen at a night temperature of 17° C was 315 gm on June 17, 772 gm on August 12, 1125 gm on September 9, and 1113 gm on October 7 (table I and fig 3). The beet roots of the plants deficient in nitrogen at a night temperature of 17° C increased in weight rather slowly, starting at 554 gm on August 12, and continuing to 675 gm on September 9 and to 871 gm on October 7. Lowering night temperatures to 10 and 4° C for the plants high in nitrogen reduced the beet root weights significantly, with the greatest decrease taking place in the beets at 4° C. Intermediate values were obtained for the plants at 10° C. Lowering night temperature for nitrogen deficient plants had very little effect on the size of the beet root for the September 9 harvest, but on October 7 the decreases were significant, with the largest decrease in the beets at 4° C. Thus it seems that a low night temperature decreases storage root growth and at the same time increases the sucrose concentration of the beet roots. These effects take place whether the beets are deficient in nitrogen or have an abundant supply of this element.

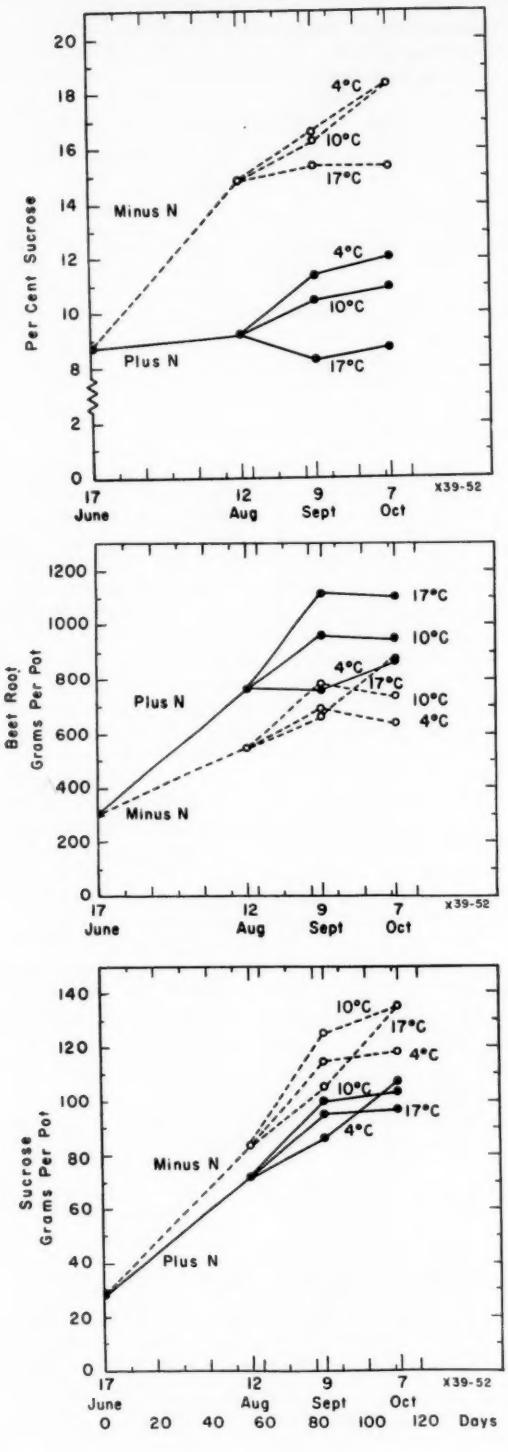
SUCROSE STORED: The sucrose stored in the beet roots at the time of harvest was calculated by multiplying the sucrose concentration of the root by its weight. The amounts of sucrose stored increased

TABLE IV

ANALYSIS OF VARIANCE OF THE EFFECTS OF TREATMENTS ON SUGAR BEET PLANTS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES							
		PETIOLE PO_4-P PPM $\times 10^{-2}$	BLADE PO_4-P PPM $\times 10^{-2}$	PETIOLE K %	BLADE K %	PETIOLE DRY MATTER %	BLADE DRY MATTER %	LEAF COUNT	
								LIVING	TOTAL
Treatments *	12	24,140	2,437,817	15.311	18.6268	64.783	18.886	359	9,748
Error *a* **	13	4,338	53,234	0.645	0.6784	0.753	1.660	25	405
Error *b* †	78	8,461	116,079	1,053	0.4782	1.135	1.293	111 ††	435

* All treatment effects relative to error *b* are significant at the 0.1 % level, except for living leaf count, which is significant at the 1 % level.
** Error *a* is the variance between trucks of the same treatment.
† Error *b* is the variance between pots within a truck. Error *b* significance is relative to error *a*.
†† Significance at the 1 % level.



with time for all plants of each treatment except for the last harvest when the minus nitrogen plants at 17° C and the plus nitrogen plants at 4° C increased more rapidly in sucrose stored than the other plants. In general, whenever a treatment decreased beet root weight (fig 3), the increase in sucrose concentration (fig 2) more than offset the loss in root size so that the sucrose stored actually increased in amount (fig 4 and table I).

At a night temperature of 10° C the sucrose stored by the beets equalled or exceeded the amounts stored at 17 or at 4° C. While the differences are not statistically significant, the trends are, nevertheless, in agreement with observations reported earlier (10). Also, a nitrogen deficiency, regardless of night temperature, resulted in an increase in sucrose stored by the plants (table I and fig 4). From the practical standpoint these observations indicate that every effort should be made to induce a nitrogen deficiency in a field of beets at the end of the season and then to harvest the crop after a period of cool weather.

Top Growth: The fresh weight of the tops was influenced mainly by the nitrogen status of the sugar beet plant. When the plants became deficient in nitrogen top growth decreased greatly, and thereafter, night temperature had little effect on the size of the tops (table I and fig 5). When the plants had an ample supply of nitrogen, low night temperature decreased top growth appreciably, particularly at 4° C, but the weights of these tops were still 2.5 times larger than the top weights of the plants deficient in nitrogen. This may be seen in the September 9 and October 7 harvests (table I).

In relation to time, the tops of the beets high in nitrogen decreased in weight gradually at all night temperatures. The highest top weight occurred on June 17 for the beets at 17° C. Thereafter the weights decreased slightly on August 12, and still more on September 9 and October 7. Decreases in top weight also took place at all night temperatures for the nitrogen deficient plants but the decreases were much smaller than for the corresponding plants high in nitrogen.

The dry weights of the tops, exclusive of the old leaves, are in general agreement with the fresh weights (table I), and like them show a major decrease with a nitrogen deficiency. Decreases in night temperature had no significant effect on the dry weights of the tops in the low or high nitrogen plants. Apparently, the increases in dry matter content of the plants at low night temperatures offset most of the fresh weight losses, which is particularly evident

Fig. 2 (*above*). Influence of nitrogen nutrition and night temperature upon the sucrose concentration of sugar beet roots. (See also fig 1.)

Fig. 3 (*middle*). Influence of nitrogen nutrition and night temperature upon the storage root growth of sugar beet plants. (See also fig 1.)

Fig. 4 (*below*). Influence of nitrogen nutrition and night temperature upon the sucrose stored within the storage root of sugar beet plants. (See also fig. 1.)

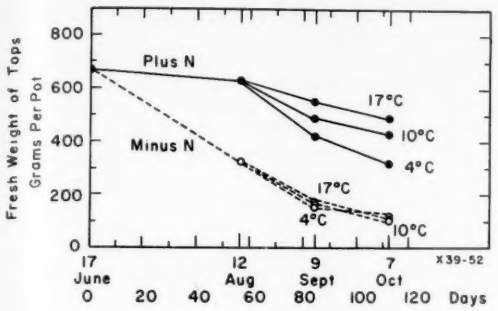


FIG. 5. Influence of nitrogen nutrition and night temperature upon the fresh weight of tops of sugar beet plants. (See also fig 1.)

in the high nitrogen plants of September 9 and October 7.

The old leaves increased in weight with the age of the plant and in relative weight at the onset of a nitrogen deficiency (table I). Once the plants have become severely deficient in nitrogen, leaf maturation takes place more slowly.

LEAF BLADE WEIGHT: In general, the fresh weight of the leaf blades followed the same pattern as the fresh weight of the tops, but the changes were somewhat less than those of the tops in relation to night temperature, nitrogen deficiency or to time of harvest (table I). The largest decrease was, again, due to a nitrogen deficiency. Lowering the night temperature for deficient plants had only a minor effect on the leaf blades. For plants well supplied with nitrogen lowering night temperature decreased blade weight considerably but the decreases were smaller than for a nitrogen deficiency.

BLADE TO TOP RATIO: The ratio of the fresh weight of the leaf blades to the fresh weight of the tops changed greatly for plants deficient in nitrogen (table I). On October 7, this ratio multiplied by 100, was 49.0 for nitrogen deficient plants at 17° C and about 32.0 for the comparable plants high in nitrogen. The latter figure is apparently characteristic of plants high in nitrogen at a night temperature of 17° C. In June this ratio was 30.8, in August 30.7, in September 30.0, and in October 31.8. A nitrogen deficiency increased this ratio to 36.3 on August 12, to 40.0 on September 9 and to 49.0 on October 7, 1952. Lowering night temperature for plants high in nitrogen also increased the ratio of blades to fresh weight of tops (table I, October 7 harvest). In both instances the increases in ratio of blades to tops appear to be at the expense of the petiole. Lowering the night temperature of nitrogen deficient plants did not increase the ratio of blades to tops appreciably. At low night temperatures the petioles of the leaves of high nitrogen plants become shorter and thicker while the blades tend to remain about the same size or even become larger. For nitrogen deficient plants both the blades and petioles decrease in size but not in number (table II), and apparently the decrease in leaf size is mainly at the expense of the petiole.

Interestingly enough, the plants with the highest blade to top ratios are also the ones with the highest sucrose concentration in the storage roots. It is hardly likely, however, that the increases in sucrose concentration of the storage roots are caused solely by the increases in blade to top ratios. Neither is the absolute amount of blade tissue solely responsible for the increases in sucrose concentration of the beet root. If the latter were true, then nitrogen deficient plants would have an exceedingly low sucrose concentration, but instead they have the highest sucrose concentrations of the plants studied. A more plausible explanation for the high sucrose concentration of the beet plants grown at low night temperatures or deficient in nitrogen is a low rate of sucrose utilization. Sucrose under these conditions would accumulate in the storage root, provided there was no comparable impairment of sucrose formation by the beet plants.

NUMBER AND HEIGHT OF LIVING LEAVES: The number of living leaves of the plants changed very little from June 17 through October 7, 1952 even though some of the plants were grown at a low night temperature and were extremely deficient in nitrogen (table II). At high nitrogen levels and at low night temperatures, the number of leaves formed decreased slightly, but the life expectancy per leaf increases at low temperatures (unpublished results), and therefore there is no appreciable reduction in the number of living leaves per plant. For nitrogen deficient plants at a high night temperature there is no significant reduction in the number of living leaves on the plant, although the number of leaves formed as shown by total leaf count (table II) decreased significantly for the October 7 plants. Accordingly, the main effect of a nitrogen deficiency appears to be a large decrease in leaf size with only a slight decrease in the number of leaves formed. Apparently, nitrogenous compounds are re-translocated to the growing point in amounts sufficient for leaf formation but not sufficient for good growth. The short height of the nitrogen deficient plants reflects a lack of growth clearly (table I).

PETIOLE AND BLADE DRY MATTER CONTENT: The dry matter content (table II) was influenced by the nitrogen status of the plants and by the night temperature. Significant increases in dry matter content of the petioles were induced by a nitrogen deficiency at all harvest dates. Lowering night temperature increased the dry matter content of the petioles of the high nitrogen plants but not the petioles of nitrogen deficient plants.

A nitrogen deficiency increased the dry matter content of the blades for some harvests (table II). Lowering night temperatures also increased the dry matter content of the blades for the high nitrogen plants but not for those deficient in nitrogen. Age of the plants had no significant effect upon the dry matter content of the blades of recently matured leaves selected for analysis at each harvest. How-

ever, leaf blades of the same plant that differ in age physiologically as well as chronologically, i.e., young vs. old leaves (not shown in the tables), do differ significantly from each other in dry matter content.

PETIOLE NITRATE-NITROGEN: The petiole nitrate-nitrogen values expressed in parts per million (ppm) on the dry basis for the plants high in nutrients and at a night temperature of 17° C remained relatively constant for all harvests (table II). The extreme values, 23,700 ppm for June 17 and 19,400 ppm for September 9 differed significantly from each other, but from the diagnostic point of view both are high in nitrogen (9). For the night temperatures of 10 and 4° C, the nitrate-nitrogen values are also high, and interestingly enough, the plants grown at the highest night temperature have the highest nitrate-nitrogen concentration. An examination of the nitrate-nitrogen and dry substance values of the petioles suggests that the nitrate values are inversely related to dry substance. This may be seen in the petiole analyses of September 9 and October 7 (table II).

The petiole nitrate-nitrogen values for the plants watered with a culture solution lacking nitrogen (table II) were well below the critical concentration at all times (9). The nitrate-nitrogen values were the highest for this group of plants at the first harvest, and thereafter, the concentrations decreased to a uniformly low level at all night temperatures. From the diagnostic point of view the uniformly low values for nitrogen deficient plants makes for greater accuracy in estimating the nutrient status of plants found below the critical concentration for nitrogen. For plants high in nitrogen the petiole nitrate values are influenced significantly with respect to time and night temperature, but here the plants are well above the critical concentration and no increase in growth would be expected by adding more nitrogen to the plants.

BLADE NITRATE-NITROGEN: The nitrate-nitrogen values for blades (table II) are much lower than for the corresponding petioles and are influenced very little by changes in night temperature, age of the plant or by nitrogen level. Still lower nitrate values for blade tissues may be obtained by removing the midribs or by selecting only interveinal tissues for analysis (unpublished results). The sharp decrease in nitrate values of blade tissues may be due to a nitrate barrier between the conducting tissues of the veins and the parenchyma cells of the blades, or to an intensive reductase activity of the parenchyma cells which prevents nitrate accumulation by the cells.

PETIOLE AND BLADE PHOSPHATE-PHOSPHORUS: The phosphate-phosphorus values for the petioles of all plants (table II) were well above the critical concentration for sugar beet plants (7). At a night temperature of 17° C the petioles in September and October were higher in phosphate-phosphorus concentration than those at 4 and 10° C. This again appears to be related inversely to dry matter content of the petioles. A deficiency in nitrogen failed to increase the phosphate content of the petioles at any night temperature.

In contrast to the phosphate-phosphorus values of the petioles those of the blades were not influenced significantly by night temperature, but instead, were affected tremendously by a nitrogen deficiency. Values as high as 30,000 ppm of phosphate-phosphorus for the blades were observed on October 7, 1952 for the nitrogen deficient plants at a night temperature of 10° C. The highest petiole phosphate-phosphorus value (8,600 ppm) also occurred on this date. The average phosphate-phosphorus value on October 7, 1952 for the nitrogen deficient blades was 18,200 ppm, and for the petioles 4,180 ppm.

PETIOLE AND BLADE POTASSIUM: The petiole potassium values (table II) were well above the critical concentration for all plants in the experiment. Raising the night temperature raised the potassium concentration of the petioles, which is similar to the observation for the petiole nitrate-nitrogen values of plants high in nitrogen. A nitrogen deficiency, except for the August 12, 1952 harvest, failed to increase the potassium concentration of the petioles. Also, the age of the plants had no consistent effect upon the potassium concentration of the petioles.

The potassium concentrations of the blades indicated again that potassium in the plant is in ample supply. Changes in night temperature produced no consistent pattern in the potassium concentration of the blades, while a nitrogen deficiency increased the blade potassium values in every instance.

ERROR WITHIN AND BETWEEN TRUCKS: The variation between pots within a truck, error *b*, (tables III and IV) was found in some measurements to be significantly greater than the variation between trucks within a treatment, error *a*. Error *b* was larger than error *a* in those measurements where positional effects, primarily light, influenced the end result, as for example, for beet root weight but not for sucrose concentration of the beet root itself. This indicates that an increase in precision can be obtained in future experiments by allowing more space between pots or in having more trucks per treatment.

SUMMARY

Beta vulgaris seed of the variety GW 304 was planted in vermiculite on February 21, 1952. The plants were grown with an ample supply of water and nutrients in a controlled temperature greenhouse (days 23° C, nights 17° C). Starting on June 17, 1952, one half of the plants were watered with a nutrient solution lacking nitrogen and the other half maintained as before on a complete nutrient solution. Beginning on August 12, 1952, one third of each set was kept at a night temperature of 17° C, another third at 10° C, and the final third at 4° C. During the day, 8 A.M. to 4 P.M., the plants were kept in the greenhouse at 23° C.

The plants high in nitrogen on August 12, 1952 had a sucrose concentration of 9.2 % and those deficient in nitrogen, 14.8 %. After eight more weeks of

nitrogen deficiency the sucrose concentrations of the beets at 17° C averaged 15.4 %, and those at 10 and 4° C averaged 18.3% with individuals as high as 20.0 %. The beets high in nitrogen at 17, 10 and 4° C, had sucrose values of only 8.8, 11.0, and 12.1 %, respectively. Ripening or "sugaring up" was induced by low night temperatures and by nitrogen deficiency, i.e., by changes in the external environment rather than by some internal self-regulatory mechanism of the plant.

The amounts of sucrose stored within the beet roots followed a pattern similar to the sucrose concentrations even though the roots of the high nitrogen plants always weighed more than their counterparts deficient in nitrogen.

A nitrogen deficiency decreased top growth of the plants greatly. Lowering night temperature decreased top growth of high nitrogen plants appreciably but had little effect on nitrogen deficient plants. Leaf blade growth followed a pattern similar to top growth. Increases in the ratio of leaf blade weight to top weight were observed for both nitrogen deficient plants and high nitrogen plants at low night temperatures.

The petioles and blades of high nitrogen plants were always higher in nitrate concentration than low nitrogen plants. The petioles of high nitrogen plants were much higher in nitrate than the blades. The petioles of high nitrogen plants at a high night temperature were always higher in nitrate than petioles of plants at a low night temperature. This was associated with the fact that the percentage dry matter of the petioles increased as the night temperature was decreased. Nitrate values for the blades were not affected by night temperature.

The phosphate-phosphorus values for the petioles were higher for the higher night temperatures and were not influenced by the nitrogen status of the plants. The phosphate values for the blades, in contrast to the petioles, were not influenced by night temperature but the values increased tremendously when the plants were deficient in nitrogen.

The potassium values of the petioles were higher, the higher the night temperature, for both levels of nitrogen. The blades of the high nitrogen plants were higher in potassium, the lower the night temperature. For the nitrogen deficient plants there was no change in potassium with night temperature. A nitrogen deficiency increased the potassium concentration of the blades but it did not increase potassium in the petioles. This was associated with the fact that the percentage dry matter of the petioles increased as the night temperature was decreased.

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COMPARATIVE PHYSIOLOGY OF GREEN AND ALBINO CORN SEEDLINGS[1,2]

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Previous comparative studies of green, albino, and variegated plants have shown higher levels of soluble nitrogenous compounds and lower levels of protein nitrogen in albino or in variegated than in green tissues (6, 14). The soluble nitrogen level was reduced and the protein level was increased when carbohydrates were supplied to albino tissues and plants. Groner (6), on the basis of such findings, postulated that when the supply of carbohydrate from the endosperm of an albino corn seedling became limiting, the seedling would hydrolyze its proteins, utilizing some of the products of hydrolysis in metabolism. The soluble nitrogenous substances, notably some amino acids, that could not be metabolized were thought to accumulate.

Respiratory studies with green and albino plants have yielded conflicting results. Lebedeff (8) reported that rates of respiration of albino corn seedlings were 17 % less than that of green seedlings. Schumacker (14) used various variegated dicotyledonous leaves and found lower respiratory rates in albino tissues. He attributed this difference to the lower reducing-sugar content of albino tissues. He found that the rate increased in both kinds of tissues when supplied with glucose. Greater increases occurred in the albino tissues. Groner (5) reported little difference in respiratory rates of green and albino corn seedlings.

It seemed desirable to investigate further the respiration and the changes of various nitrogen fractions with the possible occurrence of proteolysis in the albino corn seedlings. The findings reported here are results of two studies: (a) a time-course study of the distribution of certain nitrogenous substances in light- and dark-grown green and albino corn seedlings and (b) some aspects of the intermediary metabolism of these tissues. This study has suggested new aspects of metabolism of albino tissues, none of which were investigated completely. Consequently, this paper should be considered as a preliminary study.

MATERIALS AND METHODS

The plant material used here consisted of green individuals and "white" albino individuals of a strain of corn (*Zea mays* L.). One fourth of the seeds from a selfed, heterozygous parent had a pale-yellow marker indicating an albino germling. The pale-yellow marker and the albinism in the individuals germinating from such seeds were controlled by the homozygous recessive condition of the factor designated lw_1 (lemon-white$_1$) (13). Seeds of green and albino plants were placed in water at room temperature. This was designated as zero time of growth. After soaking 24 hours, groups of 10 seeds of each type were planted in garden loam in 10-inch pots. All seedlings were grown in a constant-temperature chamber at 25 to 26° C. Seedlings grown in light were subjected to a 12-hr photoperiod using one "white" and one "daylight" 100-watt fluorescent tube as the light source yielding 450 fc at pot level.

For nitrogen analyses, 40 plants each of the green and albino populations were harvested after 4, 6, 8, 10, 12, 15, and 20 days of growth. The seedlings were cut off as close to their seeds as possible and dried in vacuo at 65° C over P_2O_5. Each population was ground to 40 mesh in a Wiley mill. Soluble N was extracted with water from the dry powder according to Vickery et al (19). Total and soluble N were determined by a semi-micro Kjeldahl procedure using selenium oxychloride as the catalyst. (Nitrate nitrogen was not determined.) Protein N was calculated as the difference between total and soluble N. The soluble N fraction was analyzed for asparagine, glutamine, and ammonium N according to Vickery et al (18) and Pucher et al (12), and for α-amino N according to Frame et al (3). All determinations were in duplicate.

For manometric studies the expanded portions of the second and third leaves of 9-day-old green and albino seedlings were used. The leaves were split down the midvein and cut transversely with a razor blade into pieces 1 to 2 mm wide. Leaf segments from a number of plants were mixed thoroughly in distilled water, drained and placed into Warburg vessels containing 0.01 M phthalate-phosphate buffer plus 0.002 M $MgSO_4$ and 0.002 M $CaCl_2$. All solutions were adjusted to the desired pH with NaOH. Standard Warburg procedure was followed. Experiments were performed in duplicate in the dark at 26° C with the appropriate gas phase. The respiratory quotient (RQ) was obtained by a one-vessel method (10).

Etiolated plants were grown in the dark from "green" and "albino" seeds. Etiolated tissues for chemical and manometric determinations were prepared in the semi-darkened laboratory to minimize any changes in the tissues due to light. For N analyses 40 plants each of dark-grown green and albino populations were harvested after 6, 9, and 12 days of growth. For manometric studies 9-day-old, dark-grown green and albino plants were used.

RESULTS

GENERAL GROWTH HABIT: The albino seedlings used in these investigations were the same in form, but smaller than the green seedlings. Both types formed 4 leaves during the first 20 days of growth. After 12 days of growth the tips of the older leaves

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of the albino seedlings showed necrotic areas. Rates of growth of the albino plants decreased from 12 to 15 days and death occurred approximately 20 days after zero time (table I).

When etiolated, green and albino corn seedlings showed similar structural changes (fig 1). The plants became prostrate because of a relative lack of sclerenchymatous cells in the greatly elongated first internodes. Leaves of etiolated plants were longer and narrower and somewhat longitudinally curled. Etiolated "potentially green" seedlings were devoid of chlorophyll and their carotenoid pigments were visible. Etiolated albino seedlings had a slight blue-green hue which disappeared in light. Leaf material was prepared for microscopic examination from 9-day-old, light-grown plants by a freeze-dry method (7). No plastids or plastid-like bodies could be detected in the albino. Green tissues, however, had well-formed, undistorted chloroplasts.

NITROGEN METABOLISM: Total N per gram dry weight (per gm DW) (fig 2) was essentially the same in green and albino corn seedlings up to 12 days of growth. Soluble N per gm DW was the same in both tissues at 4 days of growth, increased slightly in green tissues by 6 days and then decreased. In the albino, however, soluble N content increased above that in the green seedlings at 8 days of growth, fell slightly until 12 days and then increased, paralleling the increased necrobiosis. Protein N per gm DW decreased rapidly from 4 to 6 days of growth. During the period of study the wet- and dry-weight values of the tissues increased. The rapid changes of N values from 4 to 6 days was due to a dilution through the rapid increase in non-nitrogenous dry-weight per seedling (table I).

Asparagine, glutamine, α-amino, and ammonium N per gm DW were essentially the same in both green and albino epicotyls after 4 days of growth (fig 3). Asparagine showed parallel increases in both tissues from 4 to 6 days of growth. The large

TABLE I

DRY WEIGHT IN MILLIGRAMS PER KERNEL OF LIGHT-GROWN GREEN AND ALBINO SEEDLINGS AND PER EPICOTYL OF LIGHT-GROWN AND DARK-GROWN GREEN AND ALBINO SEEDLINGS FROM ZERO TO TWENTY DAYS OF GROWTH *

	DAYS GROWTH									
	0	2	4	6	8	9	10	12	15	20
Kernels of light-grown plants										
Green	260	250	230	190	130	...	80	60	...	...
Albino	260	250	230	190	140	...	110	70	...	...
Light-grown epicotyls										
Green	...	...	8	25	46	...	66	80	82	85
Albino	...	...	8	20	33	...	46	56	61	58
Dark-grown epicotyls										
Green	...	...	...	25	...	54	...	113	...	...
Albino	...	...	...	22	...	43	...	61	...	...

* 40 seedlings per sample.

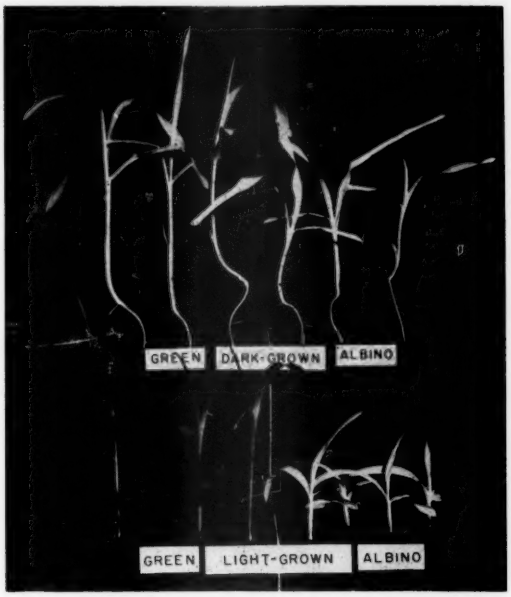

FIG. 1. Light- and dark-grown green and albino corn seedlings 9 days old.

amounts of asparagine found during early seedling development probably were synthesized from NH_4^+ absorbed from the soil in the presence of large amounts of carbohydrates from the endosperm (11). However, the accompanying decrease of glutamine N may suggest it as a source of N for the early asparagine synthesis. In the studies reported here, asparagine decreased after 6 days of growth and then increased slowly in the green seedlings, suggesting asparagine storage. After 6 days of growth, the level of asparagine in the albino remained considerably higher than that in green seedlings. The amino N per gm DW of the albino seedlings increased above that of the green and remained relatively constant throughout the period of study. Glutamine per gm DW was essentially the same in green and albino epicotyls from 4 to 20 days of growth. Asparagine and ammonia levels in albino seedlings rose sharply after 12 days of growth. The asparagine level became constant after 15 days of growth, but the ammonia level continued to rise. Thus, it appeared that during the 12 to 15 day period, ammonia released as a byproduct of protein hydrolysis and deamination, formed asparagine through amination of α-ketoglutaric acid presumably supplied via the tricarboxylic acid cycle from respirable substrate with subsequent amidation of the resulting aspartic acid. After 15 days, however, it could be that α-ketoglutaric acid was no longer available for amination. As a result, ammonia accumulated. Accordingly, asparagine formation, for a time, probably served as a detoxifying mechanism removing ammonia from the tissues (11).

There was little difference between etiolated green

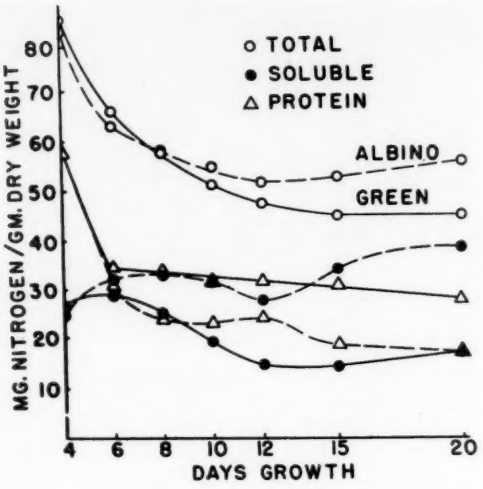

FIGURE 2

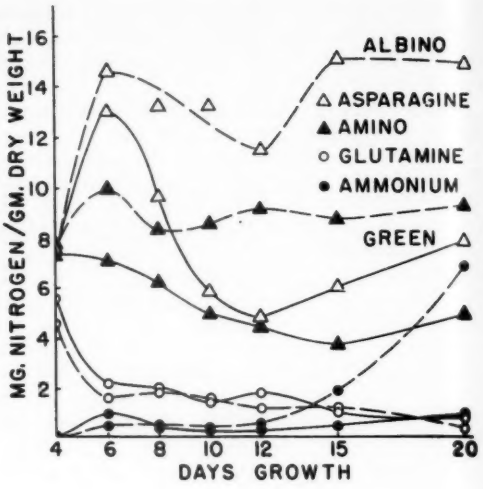

FIGURE 3

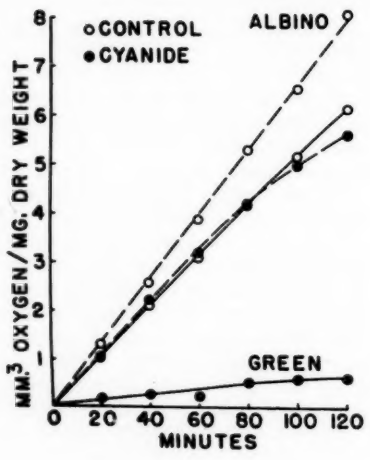

FIGURE 4

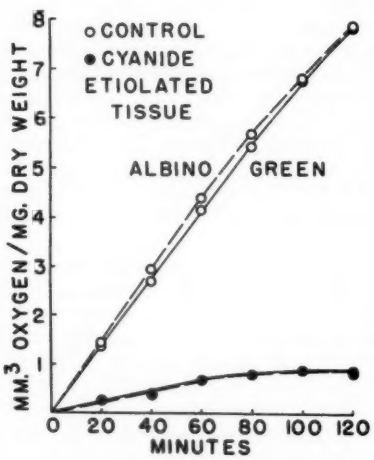

FIGURE 5

Fig. 2. Changes in total, soluble, and protein N of epicotyls of light-grown green (solid lines) and albino (broken lines) corn seedlings during the first 20 days of growth.

Fig. 3. Changes in asparagine, α-amino, glutamine, and ammonium N of epicotyls of light-grown green (solid lines) and albino (broken lines) corn seedlings during the first 20 days of growth.

Fig. 4. Effect of 0.005 M cyanide upon oxygen uptake of light-grown green (solid lines) and albino (broken lines) 9-day-old leaf tissues at pH 5.5 in the presence of 0.1 M glucose.

Fig. 5. Effect of 0.005 M cyanide upon oxygen uptake of dark-grown green (solid lines) and albino (broken lines) 9-day-old leaf tissues at pH 5.5 in the presence of 0.1 M glucose.

and albino seedlings with respect to total, protein, soluble, asparagine, glutamine, α-amino, and ammonium N per gm DW from 6 to 12 days of growth (table II). These tissues resembled the light-grown albino tissues in these particulars. Consequently, the distribution of nitrogenous compounds in light-grown albino and dark-grown green and albino tissues can probably be attributed to the lack of carbohydrate.

Asparagine or glutamine (0.005 M, pH 4.0) stimulated oxygen uptake in albino, but not in green tissues (table III). This stimulation may be indicative of asparagine and glutamine respiratory utilization in

the albino. Casein amino acids (5 mg/ml, pH 4.0) stimulated oxygen uptake in both tissues. Alanine, glycine, aspartate or glutamate (0.005 M, pH 4.0) used separately did not stimulate the oxygen uptake of either tissue. Tissue penetration of these substances was not determined.

INTERMEDIARY METABOLISM: The endogenous Q_{O_2} of 9-day-old, light-grown green leaf tissues at pH 5.5 was 2.5 to 3.0 with an RQ of 1.1. The Q_{O_2} of light-grown albino tissue was 3.0 to 3.5 (20 to 30 % higher than comparable green tissue) with an RQ of 1.0. In etiolated tissues, however, no difference in rates of oxygen uptake was evident between "green" and "albino" leaves (endogenous Q_{O_2} was 3.0 to 3.5). The same relationships occurred between green and albino tissues either light- or dark-grown in the presence of 0.1 M glucose (see green and albino controls in figs 4 and 5). Apparently green and albino seedlings differed metabolically when grown in light but not when grown in darkness.

The endogenous $Q_{CO_2}{}^{N_2}$ in 0.01 M phthalate buffer, pH 5.0, with N/CO_2 (95/5) as the gas phase, was 0.65 and 0.90, respectively, for light-grown green and albino tissues. It appeared that the metabolic dissimilarities in light-grown green and albino tissues were evident also under anaerobic conditions. Dark-grown tissues were not investigated anaerobically. It is interesting to point out the absence of a Pasteur effect in both green and albino light-grown tissues as treated here (green tissue—fermentation CO_2/respiration $CO_2 = 0.65/2.5 = 0.26$; albino tissue—fermentation CO_2/respiration $CO_2 = 0.90/3.0 = 0.30$) (Marsh and Goddard, 9).

Glucose, sucrose or maltose (0.1 M, pH 6.0) stimulated oxygen uptake in both green and albino light-grown tissues (table III). Pyruvic, citric, α-ketoglutaric and succinic acids increased oxygen uptake in the green tissues, but pyruvic and α-ketoglutaric acids were not effective in increasing respiration in the albino. Tissue penetration of these substances was not determined. Glycolic acid increased oxygen uptake in both green and albino light-grown tissues (table III) (Tolbert et al, 17).

TABLE II

NITROGEN FRACTIONS IN DARK-GROWN GREEN AND ALBINO TISSUES EXPRESSED AS MILLIGRAMS OF NITROGEN PER GRAM OF DRY WEIGHT

NITROGEN FRACTIONS	TISSUE	DAYS GROWTH		
		6	9	12
Total	Green	58.5	51.4	48.8
	Albino	59.3	57.8	52.3
Protein *	Green	26.2	20.9	22.2
	Albino	26.7	27.1	26.9
Total soluble	Green	32.3	30.5	26.6
	Albino	32.6	30.7	25.4
Asparagine	Green	14.8	13.6	11.2
	Albino	16.4	12.2	9.8
Glutamine	Green	1.8	1.6	1.4
	Albino	1.4	1.4	1.0
α-Amino	Green	7.5	8.9	8.5
	Albino	6.8	7.1	9.1
Ammonium	Green	1.5	0.7	0.7
	Albino	0.8	0.7	0.6

* By subtraction.

TABLE III

OXYGEN UPTAKE AS PERCENTAGE OF ENDOGENOUS RESPIRATION OF GREEN AND ALBINO CORN LEAVES FROM 9-DAY-OLD PLANTS, USING VARIOUS SUBSTRATES *

SUBSTRATE	CONC M	pH	GREEN %	ALBINO %	NO. OF DETERMINATIONS
Glucose	0.1	6.0	164	147	3
Sucrose	0.1	6.0	155	137	3
Maltose	0.1	6.0	149	131	2
Pyruvic acid	0.005	4.0	137	100	3
Citric acid	0.1	4.0	227	189	1
α-Ketoglutaric acid	0.005	4.5	135	100	1
Succinic acid	0.05	4.0	168	170	2
Glycolic acid	0.005	5.5	128	120	2
Asparagine	0.005	4.0	100	112	1
Glutamine	0.005	4.0	100	112	1
Casein amino acids	5 mg/ml	5.0	117	116	2

* Computed from data having less than 10 % variability.

Using malonate (0.01 M, pH 4.0) inhibition of oxygen uptake was 25 and 26 %, respectively, in green and albino tissues. The inhibition was completely reversible with 0.05 M succinate. This inhibition and its reversal may be considered as evidence for the presence of succinic dehydrogenase in both tissues. Upon addition of fluoride (0.005 M, pH 4.0) in phthalate-phosphate buffer, oxygen uptake was 51 % of controls in both green and albino tissues. Under anaerobic conditions and in the presence of fluoride (0.005 M, pH 5.0) fermentation was 37 and 31 %, respectively, of controls in green and albino tissues. The effect of fluoride upon both light-grown tissues was the same.

Light-grown green and albino tissues differed in their behavior in the presence of 2,4-dinitrophenol (DNP) (10^{-5} M, pH 5.0). Considering endogenous oxygen uptake as 100 % in green and albino tissues, respectively, oxygen uptake was 169 % in the green and 110 % in the albino tissues. With the same concentration of DNP oxygen uptake of etiolated green and albino tissues was 170 and 155 %, respectively. Thus it appeared that the respiration of light-grown albino seedlings was not stimulated by the phosphate uncoupling action of DNP (16), suggesting that they had primarily non-phosphorylative mechanisms operating.

TERMINAL OXIDATION: Azide (0.001 M, pH 4.5) inhibited oxygen uptake of green tissues 61 %, but inhibited oxygen uptake of the albino only 30 %. With cyanide (0.005 M, pH 5.5; 1.0 M cyanide in

the center well) there was 80 and 30 % inhibition, respectively, of oxygen uptake of green and albino tissues. The addition of 0.1 M glucose had no effect on this inhibition (fig 4). Studies in the dark using carbon monoxide ($CO : O_2 :: 95 : 5$) resulted in 75 and 30 % inhibition of the oxygen uptake of leaves of light-grown green and albino seedlings, respectively. Light reversal of the carbon monoxide inhibition was possible only in the albino tissues. Three attempts to demonstrate light reversal in the green tissues were unsuccessful.

Since azide, cyanide, and carbon monoxide were presumably inhibiting enzymes containing heavy-metal prosthetic groups, these data indicated that most of the oxygen uptake in the light-grown green plant proceeded via pathways mediated by such enzymes and that most of the oxygen uptake in the light-grown albino plant did not. In contrast, cyanide inhibited oxygen uptake 89 % in both green and albino dark-grown tissues under endogenous conditions or in the presence of glucose (fig 5). Azide and carbon monoxide were not tried on either of the dark-grown tissues.

Light Activation: It has been demonstrated that differences in rates of and cyanide inhibition of oxygen uptakes were not evident when the tissues were grown in darkness. To determine which wavelengths of light were operative in inducing these differences, green and albino seedlings were grown in sunlight filtered through each of three colored gelatin filters with transmissions of 445 to 490 $m\mu$ (purple), 452 to 536 $m\mu$ (blue), and 556 to 660 $m\mu$ (yellow). Differences in oxygen uptake and cyanide inhibitions, typical of light-grown tissues, occurred in the 9-day-old seedlings when the purple and blue filters were used. When the tissues were grown under the yellow filter, these differences were not observed. It would appear that the observed differences were activated by the blue-green portion of the visible spectrum. Differences in oxygen uptake between green and albino tissues were also manifested when dark-grown tissues were exposed to 24 hours of white light from a 60-watt tungsten lamp (200 fc at pot level).

Discussion

The results reported here confirm earlier findings (6) that albino corn seedlings have a higher soluble N and a lower protein N content than do green seedlings. In etiolated individuals of the higher green plant where proteolysis occurs after soluble carbohydrates become limiting, some of the amino acids formed through proteolysis may be metabolized by deamination and subsequent oxidation (1). The released ammonia can be synthesized into glutamine and asparagine so long as glutamate and aspartate are supplied via metabolic pathways. Certain amino acids and other nitrogen substances that cannot be so metabolized then accumulate. Since in this study there is little difference in the content of various nitrogenous substances in etiolated green and albino seedlings, and since these results are similar to those obtained from light-grown albino seedlings, the proteolysis hypothesis suggested by Groner for albino corn seedlings seems valid. Further evidence supporting her hypothesis is the failure of added keto acids to stimulate oxygen uptake in the albino tissues, for if large amounts of nitrogenous substances were released during proteolysis, many of these may enter into paths of carbohydrate metabolism. Where these substances are available in the albino corn tissues, additions of "physiological" concentrations of added keto acids may well have no measurable effect, for the enzyme systems are saturated by the products of proteolysis. Differential penetration of these keto acids into the tissues should not be discounted.

In the studies with inhibitors, notably cyanide and DNP, the light-grown albino seedlings show metabolic differences from the light-grown green and the dark-grown green and albino tissues. Cyanide inhibition suggests the presence of the same heavy-metal-mediated enzyme systems in all tissues. Carbon monoxide inhibition indicates the presence of the cytochrome system in both tissues. However, the role these systems play in light-grown albino seedlings is different from that in the light-grown green and dark-grown green and albino tissues. The terminal oxidation in the light-grown albino seems to be carried on by cyanide- and carbon monoxide-insensitive enzymes, probably flavo-protein in character. Further evidence is given by Eyster (2) who reports that in the albino corn seedling the activity of catalase, an iron porphyrin enzyme, is approximately one-third that of the light-grown green seedlings. When, however, the seedlings are grown in the dark, the catalase activity is the same in both green and albino tissues, but slightly higher than the light-grown green tissues. The results with DNP suggest that the respiration in the light-grown albino is primarily non-phosphorylative in character.

Because of the lack of a gene for plastid development, the albino individuals of the strain studied here lack the mechanism for development of chlorophyll and for carbohydrate synthesis. Consequently, proteolysis occurs in the light-grown albino seedlings. However, it also occurs in the dark-grown seedlings, yet their respiratory patterns differ. Therefore, the type of respiration, non-phosphorylative and cyanide insensitive, in the light-grown albino is not a consequence of proteolysis. Being unprotected by the screening action of chlorophyll the "normal" respiration, i.e., the type found in the green or dark-grown green and albino tissue, is probably destroyed or inactivated by light. The proteolysis, on the other hand, eventuates in necrosis of the tissues, possibly by the accumulation of toxic substances like ammonia released during oxidation of amides and amino acids. Spoehr (15) and Gorham (4) reported that the fatal terminus could be prevented by supplying sucrose to their albino corn seedlings. Their method apparently prevented excessive proteolysis, oxidation of its products, and accumulation of toxic byproducts.

Summary

A study of the course of changes in total, protein, soluble, asparagine, glutamine, α-amino, and ammonium N was made on light-grown and dark-grown (etiolated) green and albino corn seedlings. Soluble, asparagine, and amino N content was higher in the albino tissues. Ammonium N was the same in both green and albino tissues, but its level increased rapidly after 12 days of growth in the albino with a parallel increase in asparagine. Approximately the same values for the nitrogenous fractions, as analyzed in the light-grown seedlings, were found in dark-grown green and albino tissues.

Oxygen uptake per gram dry weight in the albino leaf tissues was 20 to 30 % higher than in the green tissues. This difference in oxygen uptake was not evident in etiolated tissues. Fermentation showed the same pattern as respiration with light-grown tissues. These results indicate the absence of a Pasteur effect in either tissue. The oxygen uptake of light-grown albino tissues is mediated by some cyanide-insensitive enzyme system, probably flavo-protein in nature, which is present when the tissues receive 24 hours of light or are grown in the blue-green portion of the spectrum. Results with DNP suggest that the metabolism of the light-grown albino corn seedlings is primarily non-phosphorylative.

The results reported are in accordance with the study reported by Groner that proteolysis occurs in the albino corn seedling.

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CARBON DIOXIDE FIXATION AND ION ABSORPTION IN BARLEY ROOTS[1,2]

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It has been known for some time that barley roots are capable of fixing relatively small amounts of CO_2 (17). More recently, Poel (18) examined the uptake and fixation of $C^{14}O_2$ in barley roots. By means of paper chromatographic techniques, he investigated the non-volatile 80% ethanol-soluble products of fixation. Malic, citric (or iso-citric), aspartic, and glutamic acids, asparagine and glutamine contained most of the C^{14} with lesser amounts in serine, tyrosine, α-ketoglutaric acid, alanine and a number of unidentified products. By pretreating the root material with tap water instead of nutrient solution, Poel obtained considerably greater uptake of $C^{14}O_2$ which appeared largely in the malic acid. On the basis of his data, he suggested that a restriction of amination as a direct consequence of nitrogen deficiency might account for the increased accumulation of C^{14} in the malic acid.

When ions are absorbed from the external solution, equivalent amounts of other ions are liberated into the solution by the roots. These ions are primarily H^+ and OH^- (or HCO_3^-) which become available in ionic form during the absorption reaction (9, 16). Unequal absorption of cations and anions by plant roots is reflected in gains or losses of inorganic ions and changes in organic acid content of the roots. This latter situation predominates in roots of young plants and malate is the anion chiefly involved (5, 9). It has been suggested that malate may be directly or indirectly implicated in the ion absorption mechanism (6, 12). It is therefore particularly pertinent to investigate the relationship between CO_2 fixation and malate metabolism in roots.

In the work to be reported here, the fixation of $C^{14}O_2$, as a function of ion absorption, was followed with particular reference to its incorporation in the Krebs cycle acids. The labeled malic and pyrrolidone carboxylic acids produced during the treatments were degraded in order to determine the intramolecular distribution of C^{14}.

MATERIALS AND METHODS

Experiments were performed with excised roots of 6-day-old barley seedlings (Sacramento variety) grown and prepared as previously described (10). Although of the same year's crop, the seed were from a different batch from that used previously (9). The composition and behavior of this root material and the earlier material were essentially similar but some quantitative differences were noted.

Absorption experiments were carried out in sealed 4.5-liter bottles using a root to solution ratio of 2 gm root material to one liter of solution. About 4 liters of test solution, previously aerated with CO_2-free air, were placed in the bottle, 9.0 gm excised roots added and the $C^{14}O_2$ solution introduced well below the surface of the test solution by means of an automatic pipette. The bottle was immediately filled with more test solution and sealed with no air space present. Previous data showed that during the absorption period, less than one-third of the dissolved O_2 was consumed. The absorption was carried out at 26.5° C for a period of three hours. During this time, the bottles were frequently turned end over end to mix the roots and solution. For each test solution, ten bottles were prepared; i.e., a total of 90 gm root material. At the end of the absorption period, the roots were removed from the solution, washed, frozen and lyophylized.

The four test solutions consisted of KBr, $CaBr_2$, KH_2PO_4 and water. The salt solutions contained 5.00 meq cation/liter. In each case, the pH of the solution was adjusted so that upon addition of the $C^{14}O_2$ solution, the initial pH would be approximately 5.0.

The $C^{14}O_2$ solution was prepared by liberating CO_2 from C^{14}-labeled $BaCO_3$ with acid and absorbing the labeled CO_2 in KOH solution. Only sufficient KOH to trap the CO_2 was used. The specific activity of the $C^{14}O_2$ preparation was 1.44×10^5 cts per sec per micromole. To each bottle was added approximately 4×10^5 cts/sec C^{14} activity. The amount of labeled K_2CO_3 added was sufficiently small, 0.5 to 0.6 micromoles/liter, so that its effect on absorption could be safely neglected. Although fairly high activities were used, the exposure periods were relatively short and no evidences of injury in either appearance or behavior were observed.

Organic acids were isolated and quantitatively determined by ether extraction followed by silica gel chromatography according to the method of Bulen et al (4). Inorganic ions were determined as previously described (9) with the exception of potassium which was determined by a flame photometric procedure. C^{14} contents were measured by "plating out" the samples to be tested for activity on a copper disc. Uniform plates were obtained by using a turntable, infra-red lamp and a trace of wetting agent. In all cases, the thickness of the "plated out" material was such that self-absorption never amounted

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to more than 2 % when calculated from the data of Libby (11). Total activities were determined from the CO_2 obtained by combustion. All samples were counted with a windowless flow counter. Analytical determinations were performed in duplicate.

Results

Fixation of $C^{14}O_2$ in Water: The fixation of $C^{14}O_2$ by barley roots in water was examined by treating the roots with water containing labeled CO_2 for a period of three hours. HBr was used to adjust the pH to approximately 5.0. The solution contained 0.58 micromoles labeled CO_2/liter and was 1.16×10^{-6} *N* in KBr. At this concentration, there is no detectable uptake of ions and the organic acid behavior is identical with that observed in distilled water. The data for this experiment are summarized in table I. The concentrations of the acids before and after the treatment as well as the concentrations of K^+ and $Ca^{++} + Mg^{++}$ are given. In addition, the C^{14} activity found in each acid, the applied dose and the total activity accumulated are given. All values are expressed on a kgm fresh weight basis.

The roots accumulated 3.11 % of the C^{14} applied in the external solution. Of this amount, 73.7 % was found in the organic acid fraction, the malic acid accounting for almost all of the activity in this fraction; i.e., 92.3 %. The identity of the acids were confirmed by autoradiographs of paper chromatographs using known acids as standards. In this way, both the inert and the radioactive components were checked. In most cases, each separated acid was free from radioactive contamination. However, because of overlapping of fractions from the silica gel column, succinic acid was usually contaminated with some pyrrolidone carboxylic acid (PCA) and the "oxalic acid" activity consisted entirely of radioactive malic acid.

Table I

The Effect of Three-Hour Water Treatment on the Organic Acid Composition and $C^{14}O_2$ Fixation in Excised Barley Roots

	Meq/kgm fresh wt		Cts $\times 10^{-4}$/sec $\times$ kgm fresh wt
	Initial	Final	
U 1 †	1.13	1.11	0.105
Acetic	7.62	8.12	0.471
Fumaric	1.36	1.37	0.347
Succinic	0.703	0.685	0.396 *
PCA	0.706	0.849	4.54
Oxalic	0.638	0.740	0.311 **
Malic	11.04	11.65	83.2
U 2 †	0.428	0.373	0.0005
Citric	0.472	0.625	0.674
Isocitric	0.243	0.254	0.0647
Total OA	24.34	25.78	90.1
K^+	13.9	13.4	....
$Ca^{++} + Mg^{++}$	8.30	7.82	....
Applied $C^{14}O_2$	....	....	3950.0
Accumulated C^{14}	....	....	123.0

* Partly PCA activity.
** Malic acid activity.
† Unidentified.

Table II

The Effect of Three-Hour Treatment with KBr Solution on the Organic Acid Composition and $C^{14}O_2$ Fixation in Excised Barley Roots

	Meq/kgm fresh wt		Cts $\times 10^{-4}$/sec $\times$ kgm fresh wt
	Initial	Final	
U 1 †	1.16	0.955	0.0790
Acetic	7.64	9.56	0.399
Fumaric	1.63	1.32	0.283
Succinic	0.665	0.878	0.449 *
PCA	0.737	0.713	3.55
Oxalic	1.09	0.765	0.494 **
Malic	10.10	12.37	80.0
U 2 †	0.346	0.414	0.0142
Citric	0.448	1.02	1.05
Isocitric	0.292	0.402	0.0758
Total OA	24.11	28.40	86.4
K^+	13.3	29.5	....
$Ca^{++} + Mg^{++}$	8.42	6.13	....
Br^-	0.03	14.0	....
Applied $C^{14}O_2$	....	....	4320.0
Accumulated C^{14}	....	....	132.0

* Partly PCA activity.
** Malic acid activity.
† Unidentified.

In this experiment, as well as in the others reported here, there were two unidentified acids, U 1 and U 2 in the tables. On the silica gel column and on paper chromatographs, acid U 1 behaved very similarly to stearic acid but probably consisted of a mixture of the higher fatty acids.

Fixation of $C^{14}O_2$ in KBr Solution: The fixation of $C^{14}O_2$ during KBr absorption was studied as described above, but in this case the treatment solution contained KBr in addition to the $C^{14}O_2$. The organic acid, K^+, $Ca^{++} + Mg^{++}$, Br^- and radioactivity data are given in table II.

During this absorption treatment, a little more K^+ than Br^- was absorbed, but the loss in $Ca^{++} + Mg^{++}$ balanced this. Of the applied C^{14} 3.05 % was accumulated by the roots during the 3-hour period, 65.4 % was present in the organic acid fraction and the malic acid activity constituted 92.7 % of the total organic acid activity. Here again the malic acid and PCA activities accounted for a large part of the C^{14} in the roots. The data obtained from this experiment were very similar to that obtained from the water treatment.

Other experiments were performed on the fixation of $C^{14}O_2$ during absorption from $CaBr_2$ and from KH_2PO_4. With $CaBr_2$, excess anion absorption occurs and organic acid content tends to decrease somewhat. With KH_2PO_4, excess cation absorption occurs and the organic acid content increases mark-

TABLE III

THE EFFECT OF THREE-HOUR TREATMENT WITH $CaBr_2$ SOLUTION ON THE ORGANIC ACID COMPOSITION AND $C^{14}O_2$ FIXATION IN EXCISED BARLEY ROOTS

	MEQ/KGM FRESH WT		CTS × 10^{-4}/SEC × KGM FRESH WT
	INITIAL	FINAL	
U 1 †	1.13	1.01	0.0458
Acetic	9.14	10.34	0.482
Fumaric	1.58	1.62	0.127
Succinic	0.663	0.487	0.0546 *
PCA	0.565	0.702	2.91
Oxalic	0.958	0.890	0.395 **
Malic	10.02	8.24	17.5
U 2 †	0.388	0.372	0.0003
Citric	0.338	0.526	0.518
Isocitric	0.412	0.447	0.0578
Total OA	25.19	24.63	22.0
K^+	15.0	14.8	....
$Ca^{++} + Mg^{++}$	8.36	9.05	....
Br^-	0.01	8.59	....
Applied $C^{14}O_2$	....	....	3620.0
Accumulated C^{14}	....	....	42.6

* Partly PCA activity.
** Malic acid activity.
† Unidentified.

edly, due almost entirely to an increase in malate content.

FIXATION OF $C^{14}O_2$ IN $CaBr_2$ SOLUTION: When roots were treated with the $CaBr_2$ solution and $C^{14}O_2$ for 3 hours, the data of table III were obtained. There was no significant change in the K^+ content, the $Ca^{++} + Mg^{++}$ went up slightly, 8.59 meq Br^-/kgm roots were absorbed. As previously shown (9), the organic acid decreased a little but this did not correspond to all of the Br^- absorbed and other inorganic cations and anions were involved. The accumulation of C^{14} by the roots during this treatment was considerably smaller than that occurring in water or KBr solution, amounting to 1.18 % of the applied dose. Smaller proportions of the accumulated C^{14} were found in the total organic and malic acid fractions; 51.8 % of the absorbed C^{14} was in the total organic acid fraction and 79.1 % of this was in the malic acid. As in the previous experiments, the bulk of the organic acid C^{14} was in the malic acid primarily and to a considerably lesser extent in the PCA. Relatively small amounts were found in the other acid fractions.

FIXATION OF $C^{14}O_2$ IN KH_2PO_4 SOLUTION: Since $H_2PO_4^-$ is very slowly absorbed by this material, absorption from KH_2PO_4 results in excess cation uptake. In young root material such as used here, a corresponding increase in the malate content of the root occurs.

Treatment with KH_2PO_4 solution and $C^{14}O_2$ for three hours resulted in the absorption of 11.5 meq K^+/kgm roots and 1.5 millimoles P; 2.88 meq $Ca^{++} + Mg^{++}$ were lost and the malate content increased by 8.53 meq. The data are summarized in table IV. More C^{14} was accumulated during this treatment than in any other treatment and the relative amounts found in the total organic and malic acid fractions were greater than in any other experiment. The root material contained 7.02 % of the C^{14} present in the test solution. Of this, 95.2 % was in the total organic acid fraction. Again the malic acid and PCA contained most of the C^{14} with 96.7 % of the total organic acid C^{14} localized in the malic acid.

DISTRIBUTION OF C^{14} IN MALIC ACID AND PCA: Since in all treatments, the major portion of the C^{14} accumulated by the roots was in malic acid and to a considerably smaller degree in PCA, these acids were degraded and the distribution of C^{14} within the acid determined.

By means of malic enzyme, malate may be oxidatively decarboxylated to CO_2 and pyruvic acid, the CO_2 arising from the β carboxyl group of the malate. This reaction can be coupled with the enzymic reduction of oxidized glutathione via glutathione reductase and catalytic amounts of TPN and Mn^{++} (13). The net reaction is

$$\text{Malate} + \text{GSSG} + H^+ \xrightarrow{\text{TPN, } Mn^{++}} CO_2 + \text{Pyruvate} + 2\,\text{GSH}$$

Labeled malate, isolated from the roots treated as described, was decarboxylated by the above system.

TABLE IV

THE EFFECT OF THREE-HOUR TREATMENT WITH KH_2PO_4 SOLUTION ON THE ORGANIC ACID COMPOSITION AND $C^{14}O_2$ FIXATION IN EXCISED BARLEY ROOTS

	MEQ/KGM FRESH WT		CTS × 10^{-4}/SEC × KGM FRESH WT
	INITIAL	FINAL	
U 1 †	0.975	0.956	0.104
Acetic	8.60	9.28	0.629
Fumaric	1.37	1.37	0.783
Succinic	0.777	1.45	0.812 *
PCA	0.622	0.553	3.87
Oxalic	0.782	0.773	1.19 **
Malic	10.70	19.23	264.0
U 2 †	0.432	0.310	0.0101
Citric	0.325	0.842	2.91
Isocitric	0.474	0.236	0.281
Total OA	25.06	35.01	275.0
K^+	13.8	25.3	....
$Ca^{++} + Mg^{++}$	8.14	5.76	....
Total P (millimoles)	10.3	11.8	....
Applied $C^{14}O_2$	....	....	4120.0
Accumulated C^{14}	....	....	289.0

* Partly PCA activity.
** Malic acid activity.
† Unidentified.

A wheat germ preparation, containing both glutathione reductase and malic enzyme, prepared by the method of Barnett et al (2) was used as the source of enzymes. The activity of the liberated CO_2 was determined and the amount of malate decarboxylated found by titration of the reduced glutathione with potassium acid iodate. Trial runs gave quantitative agreement between the malate consumed and the reduced glutathione produced.

The decarboxylation was performed in a manner essentially similar to that described by Anderson et al (1). The reaction was carried out in a suitably stoppered test tube with a side arm containing dilute sulfuric acid. At the end of the incubation period (2 hrs at 37° C), the acid was spilled into the reaction mixture and the CO_2 swept out of the acidified solution with N_2 and trapped in NaOH solution. The activity of the CO_2 was determined as previously described.

Efforts to isolate the pyruvic acid from the reaction mixture were not very successful. In general, too low and erratic results were obtained, but the data did indicate qualitatively the presence of appreciable activity in the pyruvate.

The activity of the two central carbon atoms of the labeled malate; i.e., the methylene and the carbinol carbons, were determined by chromic acid digestion. The procedure was carried out as described by Benson and Bassham (3) except on a smaller scale. The acetic acid, derived from the two central carbons of malate, was isolated by steam distillation and purified by chromatographic separation with silica gel.

The data for the two series of degradation experiments are presented in table V. From the specific activity of the malate and the amounts of reduced glutathione produced during the enzymic decarboxylation and the acetic acid produced by the chromic acid digestion it was possible to calculate the activity of the malate consumed in each case. The data in the table indicate that 46.8 to 52.3 % of the malate activity was in the β carboxyl carbon and 0.885 to 1.41 % was in the two central carbons. By difference, approximately 50 % of the malate activity was in the α carboxyl carbon. It is evident that the absorption treatment had no significant effect on the distribution of C^{14} in malate. The C^{14} was about equally divided between the α and β carboxyl groups with very little present in the remainder of the molecule.

TABLE V

DEGRADATION OF C^{14} LABELED MALIC ACID

ABSORPTION TREATMENT	CTS/SEC			
	MALIC ENZYME DECARBOXYLATION		CHROMIC ACID DIGESTION	
	Malate consumed	*CO_2*	*Malate consumed*	*Acetic acid*
H_2O	2390.0	1140.0	3580.0	50.3
KBr	2190.0	1140.0	3690.0	40.8
$CaBr_2$	1130.0	530.0	1490.0	13.2
KH_2PO_4	3300.0	1660.0	3620.0	50.3

The presence of fumarase in the wheat germ preparation would have resulted in randomization of C^{14} between the α and β carboxyl positions in malate and thus vitiate the above conclusions. This possibility was examined by adding fumarate in one run, isolating it at the conclusion of the experiment and purifying it by means of silica gel chromatography. The fumarate was found to contain approximately 0.2 % of the initial malate activity and this may have been due to contaminating traces of pyruvate. Since so little activity was trapped by the fumarate, it would appear safe to assume that practically no randomization occurred during the enzymic decarboxylation and the distribution values are reliable.

The PCA fraction was carried through a series of experiments which served both to confirm its identity and to determine the distribution of C^{14} in the molecule. The radioactive PCA fraction was mixed with inert PCA and the mixture extracted from aqueous sulfuric acid solution with ethyl acetate. The PCA recovered from the ethyl acetate was purified on a silica gel column and then hydrolized to glutamic acid with HCl. Up to this point, all of the radioactivity followed with the PCA. The glutamic acid was decarboxylated with chloramine T by the method of Cohen (7). In this reaction, the α carboxyl group was lost as CO_2 leaving β cyanoproprionic acid which was then hydrolized to succinic acid. The succinic acid was removed from the reaction mixture by the silica gel chromatographic procedure and its activity determined. Only 0.87 % of the initial activity was found in the succinic acid. This indicated that almost all of the C^{14} was in the CO_2 liberated and therefore the C^{14} was localized in the carboxyl group of the PCA.

DISCUSSION

The results indicate that CO_2 fixation is a normal process of young barley roots and its magnitude is determined largely by the concurrent ion absorption treatment. Fixation occurred in water, during equivalent cation and anion uptake, excess cation uptake, and excess anion uptake. It occurred to the greatest extent during excess cation uptake when malate content increased and to the least extent during excess anion uptake when malate tended to decrease. About six times as much $C^{14}O_2$ was fixed in the former case as compared to the latter. The $C^{14}O_2$ fixed in water and during equivalent cation and anion uptake were very similar. Approximately 45 % as much $C^{14}O_2$ was fixed in these two cases as was fixed during excess cation uptake. It is apparent from this that the differing values of CO_2 fixation reported by Poel (18) are more probably related to the extent of excess cation absorption occurring rather than to the nitrogen status of the plant material. Since he used phosphate buffer as a supporting media, excess cation absorption presumably took place during his experiments. The magnitude of this absorp-

tion would be affected by the treatment the roots received prior to the experiment. Although Poel reported the presence of several radioactive amino acids, only glutamine was detected in the present study.

The data strongly suggest that the malate is produced in large part by a carboxylation reaction. This may well be the reductive carboxylation of pyruvate via malic enzyme or the oxalacetic carboxylase reaction followed by reduction to malate. In either case the malate should be preferentially labeled in the β carboxyl position. The malate was found to be preferentially and about equally labeled in both the α and β carboxyl positions. This observation does not exclude the participation of malic enzyme or oxalacetic carboxylase in the malate synthesis since the labeled carboxyl group may become randomized between the α and β positions of the newly formed malate through equilibration by cellular fumarase.

Since only small amounts of CO_2 from the external solution are fixed, it would be necessary that substantial amounts of endogenous CO_2 be used in the carboxylation reaction to account for the malate increase during excess cation absorption. The data of Ordin (15) and Milthorpe and Robertson (14) indicate that barley roots consume 10 to 15 millimoles O_2/kgm fresh wt $\times$ hr. This rate of respiration would be more than adequate to provide the required amounts of CO_2.

Although the PCA accumulated little C^{14} compared to malate, it was the next most radioactive acid present and contained much more C^{14} than the remaining acids. However, the conditions of extraction were such that the PCA might have been produced from glutamine present in the tissues. When aqueous extracts of lyophylized root material were examined by means of paper chromatography and subsequent autoradiography, it was clearly evident that the PCA found was produced from glutamine originally present.

Since the carboxyl group of the PCA contained essentially all of the C^{14}, the parent glutamine must have been labeled in the α carboxyl position. It seems likely that the labeled glutamine was produced from labeled α-ketoglutarate, although the latter was not detected in these experiments. The observed labeling of the glutamine would require the α-ketoglutarate to be labeled in the α carboxyl position. This labeling could arise either from the labeled malate via the Krebs cycle or by enzymic equilibration of $C^{14}O_2$ with α-ketoglutarate as reported by Goldberg and Sanadi (8).

Considerably more C^{14} was accumulated in malate during excess cation absorption and considerably less during excess anion absorption when compared to that accumulating as the result of malate turnover during the water and KBr treatments. Quantitatively, the correspondence between the C^{14} found in the malate and the change in malate concentration was poor. This may have been due to a lack of constancy in the ratio of the endogenous CO_2 to the labeled CO_2 fixed.

Summary

When supplied with labeled CO_2 in solution, young excised barley roots fixed from 1.18 to 7.02% of the CO_2 in three hours. The smaller figure was obtained during excess anion absorption and the larger during excess cation absorption. In water and during equivalent cation and anion absorption, intermediate amounts of CO_2 were fixed. In all cases, malate activity accounted for a large portion of the C^{14} present in the tissues. This was particularly marked in the case of excess cation absorption with 92% of the accumulated C^{14} in the malate. Even in water, malate turnover accounted for 68% of the total accumulated C^{14}. In all treatments, the malate was preferentially and approximately equally labeled in the α and β carboxyl positions.

Glutamine, which appeared in the analysis as pyrrolidone carboxylic acid, contained very much less C^{14} than the malate, but considerably more than any other identified constituent. The glutamine was labeled almost exclusively in the α carboxyl position.

The evidence suggests that carboxylation plays an important role in the synthesis of malic acid in young barley roots.

The author gratefully acknowledges the advice and assistance of Dr. E. E. Conn in the enzymic decarboxylation of malic acid.

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SHORT-TIME FIXATION OF LABELLED CARBON DIOXIDE BY BARLEY LEAVES UNDER STEADY STATE CONDITIONS[1,2]

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Kinetic analysis of the formation of intermediates in a rapidly operating biochemical system demands close control of all variables such as light, temperature and composition of gases to which the tissue is exposed. When isotopic tracers are used for studying the kinetics of the production of photosynthetic intermediates, it must be possible to change from non-labelled to labelled gas without disturbing steady state conditions, or alternatively to shift rapidly to a new state if one desires to study the effect of such a shift. Systems to accomplish these changes have been devised by Bassham et al (1) and by Calvin and Massini (2) for the study of the kinetics of $C^{14}O_2$ fixation by algal suspensions and also for tests with soybean leaves. This paper will describe a system used to expose barley leaves to labelled gas for as short a time as 0.10 ± 0.03 seconds under steady state conditions.

MATERIALS AND METHODS

For plant material, the first leaves of barley, *Hordeum vulgare*, var. Oderbrucker were cut 6 to 10 days after planting. Six leaves were weighed, tied together near their base and put into chamber F of the apparatus shown in figure 1, where they are illuminated continuously at about 2000 fc by a 150-watt reflector flood lamp. The solenoids at A and A′ were opened by energizing them on throwing S_3 of the control circuit (fig 2) to the "cold" position. Non-labelled gas from bottle 1 then was circulated over the tissue by the pump, an auto fuel pump driven by a cam turned by an electric motor. After 10 to 20 min, S_3 was thrown to the "hot" position; this caused solenoids A and A′ to close and B and B′ to open and allowed gas, containing $C^{14}O_2$, to circulate over the tissue from bottle 2. If a very short exposure to $C^{14}O_2$ was desired, switch S_2 was closed before S_3 was moved to the "hot" position. Then as soon as S_3 was closed C_2 began to charge through R_2. When the voltage across L_1 reached the pull-in voltage, the solenoids at B and B′ were de-energized and those at C and C′ were energized; this allowed boiling water in chamber E (under pressure from a nitrogen tank attached at D) to force the leaves into boiling 95 % ethanol in G. (In our experiments, the extract obtained in flask G either was analyzed by paper chromatography or was dried for counting.) In experiments longer than 5 seconds, the upper time limit for reliable operation of the RC circuit, S_2 is closed after S_3 and the interval between these operations measured by a stopwatch.

This sequence of events was photographed with an 8 mm motion picture camera set at 60 frames per sec (film speed determined by frame count versus movement of a stopwatch placed in front of the camera). Both solenoids of a pair (e.g., C and C′) were found to open at the same instant; if both were partly open in a frame, they were open the same amount and were completely open in the next frame. Opening required less than the time for exposure of one frame (less than 0.017 sec). Solenoids B and B′ opened 1/60 to 3/60 sec after A and A′ closed depending on how vigorously switch S_3 was pushed. Solenoids C and C′ lagged B and B′ by less than 1/60 sec indicating very rapid operation of L_1.

By substituting bromine for $C^{14}O_2$ in bottle 2 it was possible to measure the delay in appearance of gas from bottle 2 in chamber F after opening B and B′. This delay always was 4/60 sec, which agrees

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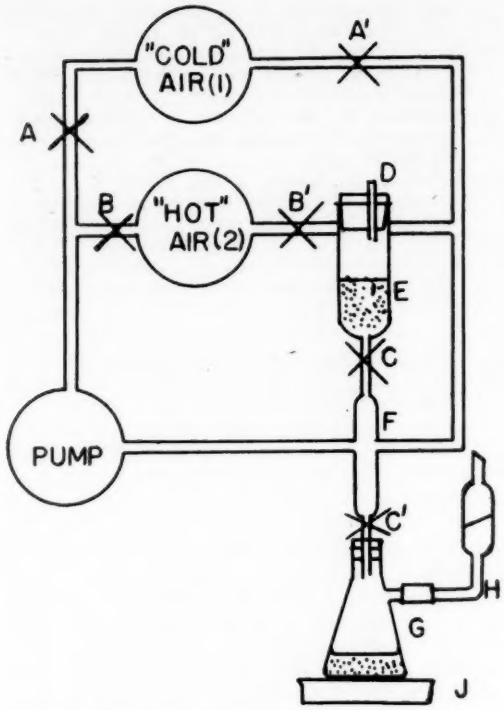


FIG. 1. Diagram of the gas circulating system and killing system used for steady state photosynthesis.

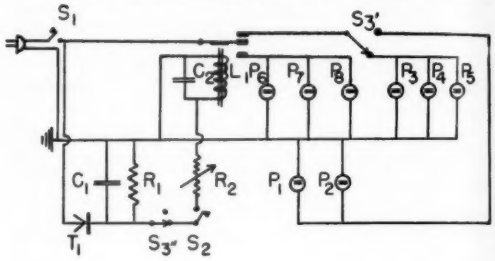
A, A' - "cold" solenoids.
B, B' - "hot" solenoids.
C, C' - "kill" solenoids.
D - inlet for 15 lb air pressure.
E - 35 ml water boiling at the rate of 121° C.
F - $C^{14}O_2$ exposure chamber.
G - 500 ml suction flask containing 150 ml boiling 95 % ethanol.
H - "Iowa" type splash trap.
J - hot plate.

closely with the delay calculated from the volume of tubing between bottle 1 and F and the rate of gas flow. The barley was completely covered with hot water in 3/60 sec after solenoids C and C' were opened. F was empty 12/60 sec after this time. Since $C^{14}O_2$ from bottle 2 does not reach F until 4/60 sec after B and B' are opened and is not washed out by water until 3/60 sec after they are closed, the shortest feasible exposure to $C^{14}O_2$ is 4/60 sec—1/60 sec before C and C' open and 3/60 second after. For such exposures the control, R_2, must be set to give a 5/60 sec interval between opening of the B and the C solenoids.

As gas flow in the exposure chamber F is turbulent, it may take longer than the gas replacement time of 0.075 sec to distribute the $C^{14}O_2$ uniformly in the chamber. With longer exposures 0.10 sec should be subtracted from the elapsed time figure (time the B solenoids are energized) to correct for this lag in equilibration of the gas. In times shorter than 0.4 sec the ratio of C^{14}/C^{12} may be computed by assuming that turbulent flow replaces 63 % of the original gas each time a chamber volume of gas passes through it.

The time the B solenoids are energized is measured by attaching the set of relays diagrammed in figure 3 across the "count" switch in the usual scaler employed with a Geiger counter. This scaler is arranged so that when the switch is open, pulses impressed on the input from a 60-cycle pulse generator will be recorded. Therefore the relay circuit is arranged to give an open circuit at P_3 (fig 3) by closing L_1, when the B solenoids are energized, and shorting the input at all other times. The frequency of the pulse generator is determined by that of the AC line, and time is thus recorded on the scaler in intervals of 1/60 sec.

Figure 4 is a photograph of a solenoid used in



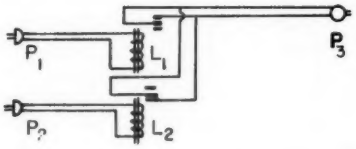


FIG. 2 (*above*). Schematic diagram of control box circuit used for controlling the solenoids of the apparatus shown in figure 1.

S_1 - "on-off" switch SPST.
S_2 - "kill" switch SPST.
S_3 - "hot-cold" switch SPDT.
P_1 and P_4 - plugs for "cold" solenoids, A and A'.
P_3 and P_5 - plugs for "hot" solenoids, B and B'.
P_6 and P_8 - plugs for "kill" solenoids, C and C'.
R_1 - 20,000 ohms, 1 watt.
R_2 - 20,000 ohms, 4 watt wirewound.
C_1 - filter capacitor, 5 μfd.
C_2 - delay capacitor, 116 μfd.
T_1 - selenium rectifier, 150 ma.
L_1 - relay, Potter Brumfeld LM-5, 2500 ohms.

FIG. 3 (*below*). Diagram of the relay system employed to activate the scaler used to measure the time leaves were exposed to $C^{14}O_2$.

L_1, L_2 - SPDT 110 v, 2.5 amp relays.
P_1 - plugs in P_5 of fig 1.
P_2 - plugs in P_8 of fig 1.
P_3 - plugs in scaler outlet.

this apparatus. The throw of the armature may be changed by moving the bar against which the blade strikes, and the spring tension holding the blade against the bar when the solenoid is closed may be varied. The solenoid, a Model 4 PPW manufactured by Allen-Bradley Company, will open against sufficient spring tension to close a ¼" bore, ⅛" wall gum rubber tube under 30 lbs air pressure (about 15 lbs pull on the armature).

Results and Discussion

Table I shows the amount of C^{14} fixed in different periods by barley leaves exposed to gas containing 0.035 % CO_2 with 9 % C^{14}. The variation in rate of fixation was rather large, but in other work (3) with larger samples of barley this variation was no more than 5 %. Probably there is considerable difference in rate of assimilation between individual barley leaves. Other experiments show that the rate of photosynthesis varies greatly with age of the barley leaf, being highest when the leaf is elongating most rapidly.

The general agreement in rate of C^{14} uptake between very short exposures and longer ones indicates that the very early products of CO_2 fixation are stable to boiling in hot ethanol. Radioautographs of paper chromatograms showed all the detectable radioactivity to be in phosphoglyceric acid after the shortest exposures to $C^{14}O_2$.

In addition to providing a means for carrying out kinetic studies of photosynthesis, the electrical controller will serve to duplicate timed experiments to very close limits. With appropriate modifications of the glassware, reactions between any two liquids or gases may be run for a definite time and brought to an abrupt conclusion.

Table I

Photosynthetic Fixation of $C^{14}O_2$ by Barley Leaves under Steady State Conditions

Time of exposure to $C^{14}O_2$	C^{14} recovered in 80 % ethanol extract	Rate of fixation of C^{14}
sec	*cts/min* *	*cts/min* **
154.0	....	12,600
21.0	4580	13,110
10.0	1538	9,230
1.13	202	10,720
.48	65.6	8,130
.52	92.4	10,610
.31	48.7	9,250
.250	56.6	13,600
.250	44.2	10,600
.117	21.3	11,600
.100	24.9	14,900
Average rate		11,300

Barley leaves were 7 days old. Gas contained 0.035 % CO_2 with 9 % C^{14}. Light was 2000 fc from a 150 watt reflector flood lamp.

* For 50 mg fresh barley.

** In extract of 50 mg fresh barley per min fixation $C^{14}O_2$.

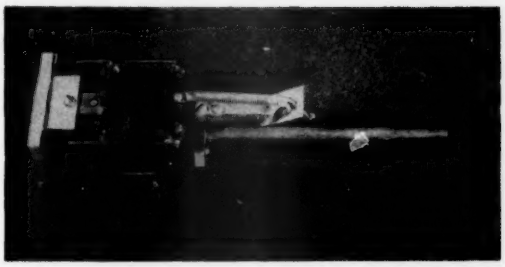


Fig. 4. Photograph of the 4-PPW solenoid modified by attachment of jaws and closing spring.

Summary

A device is described which will expose barley leaves to $C^{14}O_2$ for times as short as 0.10 ± 0.03 sec before inactivating the tissue rapidly in boiling water and ethanol. The rate of fixation of $C^{14}O_2$ into an alcohol extract of barley leaves remained essentially constant over a range of 0.10 to 154 sec. Phosphoglyceric acid was the only detectable labelled compound in the two shortest exposures.

The author wishes to express his appreciation to Prof. R. H. Burris, in whose laboratory this work was done and to Prof. M. J. Johnson, without whose technical advice it would not have been done.

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STUDIES IN PLANT METABOLISM. V. THE METABOLISM OF RADIOACTIVE 2,4-D IN ETIOLATED BEAN PLANTS[1,2,3]

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Radioactive 2,4-dichlorophenoxyacetic acid (2,4-D) has been used to study the translocation and metabolism of this chemical in plants by several workers (1, 2, 3, 4, 10, 11). Holley et al (2) and Holley (3) using carboxyl-C^{14}-labeled 2,4-D demonstrated the presence of free 2,4-D and two radioactive water soluble, ether insoluble organic acids in bean plant homogenate. Jaworski and Butts (4), using both carboxyl- and α-methylene-C^{14}-labeled 2,4-D, found that two major radioactive compounds in addition to unchanged 2,4-D were present in an 80 % alcohol extract of the stems of bean plants after treatment on the leaf with radioactive 2,4-D. One of the major radioactive compounds was found to be a complex containing 2,4-D. The formation of this complex was suggested to be a detoxification mechanism in bean plants.

Mitchell and Brown (5) found that 2,4-D was not translocated from the leaves of young bean plants which had been depleted of carbohydrates. Rice (7) was able to demonstrate that even though transport of 2,4-D from carbohydrate-depleted leaves did not occur, entry of this chemical into the leaf did take place. It has been further shown by Rohrbaugh and Rice (8), and Weintraub and Brown (9) that carbohydrate-depleted bean plants can be induced to move 2,4-D stimulus from the leaves by applying sucrose, glucose, fructose, maltose and galactose to the leaves. Later, Mitchell et al (6) using normal plants demonstrated that the translocation of 2,4-D was increased by application of sugar solution externally to the leaves. Addition of boric acid to the sugar solution will increase the rate of translocation of 2,4-D by 50 % as compared with the sugar solution alone. They found that glucose was the most effective sugar, followed by sucrose and fructose.

The present experiments were undertaken in an attempt to obtain direct information concerning the effect of sugars and other chemicals on the translocation and metabolism of 2,4-D in etiolated plants.

EXPERIMENTAL METHODS

Sixty bean plants (*Phaseolus vulgaris* var. Black Valentine) were grown in individual pots in a dark box for a period of 12 days, and a second group of 8 plants was grown under greenhouse conditions for the same length of time. The temperatures of the greenhouse were 70 to 75° C during the daytime and approximately 60° C during the nighttime.

The plants which had been grown under greenhouse conditions had fully unfolded primary leaves and were of normal height, 5 to 6 inches. On the other hand, the etiolated plants were much elongated (7 to 8 inches height) with underdeveloped leaves. The size of the primary leaves of the etiolated plants were much smaller and the petioles were much shorter as compared to those of the normal plants. The trifoliate leaves of both normal and etiolated plants were still folded in the terminal buds.

Four plants were selected at random to comprise a group of each of the 16 treatments. All plants were treated with 20 μg of carboxyl-C^{14}-labeled 2,4-D acid per plant. A 95 % ethanol solution containing 0.1 % radioactive 2,4-D and 0.5 % Tween-20 was used. 2,4-D solution was applied to the base of one primary leaf by means of a microsyringe. In order to eliminate the difficulty of immersing a proper amount of leaf area into the solution, the following technique was used. A thread, 2 inches in length, ran through the midrib of the treated leaf (approximately 1 cm from the point received 2,4-D treatment), with the lower end dipping into a vial containing 5 ml of one of thirteen solutions. Through capillary action, the treated leaf was getting a continuous supply of the chemical from the solution. The vials were filled daily with the proper solutions. The solutions supplied to the leaves contained 0.025 % sulfanilamide for the purpose of minimizing microbial growth. All plants, except the normal ones, were kept in the dark for 4 days following treatment. All manipulations were carried out at night under diffused red light.

Four days after treatment, each group of plants was harvested by cutting off the plant level with the soil. The plants were sectioned into leaves and stems (including petioles and terminal buds), homogenized separately in a Waring blendor with 80 % ethyl alcohol for 1 minute and transferred quantitatively to suitable volumetric flasks.

The radioactivity of the extracts was determined by the direct plating method. Five-tenth ml samples were evaporated to dryness in 1 inch stainless steel cupped planchets and counted with a thin mica window G-M counter (1.9 mg/cm^2) and a Tracerlab "64" scaler. In every case an accuracy of 5 % or better was realized. For the identification of 2,4-D and other radioactive compounds, the extract was applied to a spot about 7 cm from the end of strips of Whatman #1 filter paper (1 × 22 in.) by means of a fine eye dropper. Two strips were made in each case. All chromatograms were developed in a n-butanol-propionic acid-water (12 : 5.6 : 8) system for 12 hours. A descending chromatographic technique was used.

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[4] Present address: Monsanto Chemical Company, St. Louis, Missouri.

After developing, the strips were air dried at room temperature and cut into 1-cm sections starting from the original spot. The radioactivity of each section was determined directly. The relative percentage concentrations of compounds containing C^{14} in the 80 % alcohol extracts were computed (1, 4).

Results and Discussion

The data on the radioactivity recovered from the leaves and stems of all 16 groups of bean plants are presented in table I. In the etiolated plants, no radioactivity was found in the stem extract from plants which received no supplement revealing that 2,4-D was not translocated to the stem. In the normal plants, approximately 35 % of the applied activity was translocated from the leaf. The accumulation of C^{14} in the bean stems was increased with an exogenous supplement of sugar solution to the treated leaves. This observation confirms the work of Rohrbaugh and Rice (8) and of Weintraub and Brown (9), that the movement of 2,4-D from the leaves of carbohydrate-depleted bean plants can be induced by applying certain sugar solutions to the leaves even though the plants were kept in darkness. Sucrose and glucose were most effective among the carbohydrates tested, followed in order by melibiose, fructose, cellobiose and galactose. Gentiobiose, arabinose, succinate, glucose 1-phosphate and phosphoglycerate were relatively ineffective. The non-specificity with respect to sugar suggests that transport of radioactive 2,4-D is facilitated by food movement in general rather than by any specific combination with particular metabolite. Since both glucose and glucose-1-phosphate are common constituents in plant tissue, the ineffectiveness of glucose-1-phosphate in particular suggests that 2,4-D may inhibit the enzyme system involved for the conversion of glucose-1-phosphate to glucose in treated plants. From a separate identical experiment, using P^{32}-labeled glucose-1-phosphate, we were able to demonstrate the presence of radioactivity beyond the point where the thread entered the leaf. A small amount of radioactivity was also noted in the petioles and the terminal buds of the plants. It is believed in this case that glucose-1-phosphate was taken up by the plants by this method even though it is known that glucose phosphate esters do not penetrate readily the intact cells.

The average values of chromatographic results of 80 % alcohol extract of bean leaves are represented in table II. Due to the low radioactivity found in most stem extracts, only four groups were analyzed chromatographically. In general, two major radioactive spots were found in all chromatograms. One radioactive spot, having a R_f value of 0.84 to 0.88 was free 2,4-D and the other spot, R_f 0.44 to 0.46, was a 2,4-D complex. Three additional radioactive compounds were found to be present in many of the extracts but in very low concentrations. Their respective R_f values were 0.22 to 0.28, 0.36 to 0.38, and 0.65 to 0.68. In this experiment, the R_f value (0.44) of 2,4-D complex (unknown 1) was found to be somewhat higher than the value reported previously (4). A slight increase of the amount of water in the solvent composition and the use of a different batch of filter paper strips may be responsible for the change of R_f value.

Under identical conditions, no significant difference in the rate of reaction between 2,4-D and plant substrate (formation of unknown 1) in the leaves of etiolated and normal bean plants was observed. It is believed therefore, that the metabolism of radioactive

Table I

Absorption and Translocation of C^{14} in Etiolated Bean Plants Treated with 20 μg Radioactive 2,4-D and Supplemented with Different Chemical Solutions

Plant used	Treatments	Activity recovered in leaves	Activity recovered in stems	Total recovery	Translocation and accumulation in stems
		$\times 10^3$ cpm	$\times 10^3$ cpm	%	%
Normal	None	65.6	60.5	74	35.2
Normal	0.025 % sulfanilamide	84.2	50.4	79	29.4
Etiolated	None *	122.7	0.0	96	0.0
"	0.025 % sulfanilamide *	128.7	0.8	101	0.62
"	1 % sucrose	150.0	11.7	94	6.80
"	1 % glucose	155.0	5.5	94	3.20
"	1 % fructose	150.1	2.2	89	1.28
"	1 % galactose	163.9	1.7	97	0.99
"	1 % arabinose	170.0	0.5	99	0.29
"	1 % cellobiose	143.8	1.9	85	1.11
"	1 % melibiose	125.5	2.6	75	1.51
"	1 % glucose 1-PO_3	159.3	0.5	93	0.29
"	1 % ph-glycerate *	113.9	0.2	89	0.16
"	1 % succinate	173.5	1.1	102	0.64
"	1 % indoleacetic acid	163.5	0.8	96	0.47
"	1 % gentiobiose	156.8	0.9	92	0.52

Each group consisted of 4 plants.
* These groups consisted of 3 plants.
(1 μg 2,4-D = 2160 cpm)

TABLE II

PERCENTAGE DISTRIBUTION OF MAJOR RADIOACTIVE COMPOUNDS FOUND IN 80% ALCOHOL EXTRACTS OF LEAVES AND STEMS FROM BEAN PLANTS TREATED WITH CARBOXYL-C^{11}-LABELED 2,4-D AND SUPPLEMENTED WITH DIFFERENT CHEMICAL SOLUTIONS

PLANT USED	TREATMENT	PLANT PART	R_f VALUES OF RADIOACTIVE SPOTS	
			COMPLEX OF 2,4-D 0.44–0.46	FREE 2,4-D 0.84–0.88
			%	%
Normal	None	leaf	24	68
Normal	0.025% sulfanilamide	leaf	24	73
Etiolated	None	leaf	25	71
"	0.025% sulfanilamide	leaf	33	56
"	1% sucrose	leaf	29	63
"	1% glucose	leaf	33	62
"	1% fructose	leaf	30	66
"	1% galactose	leaf	24	67
"	1% arabinose	leaf	23	72
"	1% cellobiose	leaf	27	51
"	1% melibiose	leaf	28	60
"	1% glucose 1-PO_3	leaf	31	53
"	1% ph-glycerate	leaf	24	67
"	1% succinate	leaf	24	69
"	1% indoleacetic acid	leaf	20	71
"	1% gentiobiose	leaf	35	55
Normal	None	stem	68	23
Normal	0.025% sulfanilamide	stem	74	17
Etiolated	1% sucrose	stem	45	43
Etiolated	1% glucose	stem	38	57

All values are average of duplicate runs.

2,4-D will take place equally well in both etiolated and normal plants.

Mitchell and Brown (5) have reported that no stem curvature occurred in starch-depleted bean plants which were treated with 2,4-D on the primary leaf and kept in darkness. If the treatment were made on one side of the stem, the curvature developed within 24 hours. They explained that the failure of the stem of starch-depleted bean plants to develop curvature was due to the insufficient amount of 2,4-D translocated to the stem. In the present experiment, no stem curvature was observed in all etiolated plants. For the same reason, the failure of etiolated plants to develop a stem curvature might also be due to the insufficient amount of 2,4-D in the stem. However, to clarify this question, eight etiolated plants were raised in the same manner. Radioactive 2,4-D solution (20 μg per plant) was applied to the terminal bud, to the stem or to both. Again, no stem curvature was noted 4 days after treatment. At this time, the plants were harvested and the stems were homogenized with 80% ethanol. Chromatographic results revealed that this extract contained 33% of the applied 2,4-D as a 2,4-D complex and the remaining was free 2,4-D. The failure of the stems of etiolated plants to respond to 2,4-D treatment could not therefore, be explained either by the lack of 2,4-D in the stem or at a vital site of the plant, or by the absence of 2,4-D complex. The formation of a 2,4-D complex is independent of the photosynthetic processes as well as the influence of a number of exogenous metabolites. It also appears that the occurrence of a physiological response by 2,4-D treatment is dependent on the presence of certain enzyme systems requiring activation by light.

SUMMARY

A study has been made on the translocation and metabolism of carboxyl-C^{14}-labeled 2,4-D by etiolated bean plants. It was found that the growth regulator was absorbed by the leaves of etiolated plants. The movement of the 2,4-D molecule or 2,4-D complex from the leaf to the stem in etiolated plants may be induced by applying a sugar solution to the leaves. Sucrose and glucose were most effective among the chemicals tested.

Radioactive 2,4-D is metabolized in the leaves of etiolated plants as well as in the normal plants. Under identical conditions, no significant difference in the rate of reaction between 2,4-D and plant substrate (formation of 2,4-D complex) in the leaves of etiolated and normal bean plants was observed.

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PHOTOSYNTHESIS AND RESPIRATION OF THREE BLUE-GREEN ALGAE[1,2]

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Reported herein is a study of certain salient features of photosynthesis and respiration in three blue-green algae, *Anabaena variabilis*, *Anacystis nidulans*, and *Nostoc muscorum* G. Histories and brief descriptions of the three species are included in a previous report (2) which presented methods of culture and growth characteristics. Of the various blue-green algae available in pure culture the species used were chosen because they suspend readily to give homogeneous suspensions which submit to ease of manipulation. The objectives of the study are two-fold: first, to obtain information on metabolism of the blue-green algae, a group which has received only cursory attention; and secondly, to develop a base of information which would allow use of a blue-green alga as a reliable experimental organism for studies on the process of photosynthesis. Because of the simplicity of their structure the blue-green algae appear especially desirable for studies on photosynthesis pursued to subcellular levels.

Methods

Cellular material of *Anabaena variabilis* and *Anacystis nidulans* was obtained from bacteria-free cultures grown in a continuous-culture apparatus (6). The culture medium was medium C, described completely in a previous report (2), containing per liter 0.25 gm $MgSO_4 \cdot 7\,H_2O$, 1.0 gm K_2HPO_4, 0.025 gm $Ca(NO_3)_2 \cdot 4\,H_2O$, 1.0 gm KNO_3, 0.165 gm sodium citrate, 0.004 gm $Fe_2(SO_4)_3 \cdot 6\,H_2O$, and 1.0 ml of an A_5 trace element solution; pH 7.3 to 7.5 when aerated with 0.5 % CO_2 in air. The cultures were maintained at 25 or 39° C and illuminated by tungsten lamps. Pure cultures of *Nostoc muscorum* G (strain obtained from G. C. Gerloff) were grown with the same medium and aerating gas in large test tube cultures in baths held at 25° C and illuminated by 20-watt daylight fluorescent lamps. For all cultures rate of growth was controlled by light intensity. However, since the illumination was multidirectional and the effective light intensity not easily interpretable, a more useful description of the previous history of the cells is provided by the specific growth rate, k, which will be expressed in $\log_{10}$ units per day.

Photosynthesis measurements were made by Warburg manometry in a water bath with glass bottom thermostated at 25 or 39° C. Illumination was provided by a bank of tungsten lamps operated at 120 ± 1 volts from a voltage stabilizer. Light intensity could be varied by use of Jena NG neutral filters attached to the bottoms of the vessels by holders which also masked out stray light. Respiration measurements were made in conical flasks containing 2.0 ml cell suspension, 0.25 ml KOH in the center well, and 0.5 ml of buffer or buffer plus substrate in the sidearm.

In preparation for manometric experiments cells were centrifuged out from a known volume of suspension, washed in the buffer to be used, and taken up in buffer. Because of its small size (ca $1.6 \times 2.2\ \mu$) *Anacystis nidulans* does not sediment as easily as other algae; $3300 \times g$ for 6 minutes in an angle centrifuge were required for complete sedimentation. Cell quantity was determined routinely in terms of packed cell volume. Aliquots of original cell suspension were centrifuged out, transferred to calibrated capillary tubes, and centrifuged at $2400 \times g$ for one hour in an International centrifuge. Packed cell volume of all three species attained a minimum volume after 45 minutes. The relation between cell volume and dry weight was determined separately upon several differ-

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TABLE I

CHARACTERISTICS OF THE CELLS USED

ALGA	LIGHT INTENSITY FOR GROWTH	TEMP	pH	GROWTH RATE, K	DRY WT *
	fc	*°C*			*mg/cm cells*
Anabaena variabilis	55	25	7.8	0.30	0.206 ± 0.005
Anacystis nidulans	75	25	7.7	0.55	0.278 ± 0.006
	90	39	7.8	1.00 †	0.271 ± 0.007
	220	39	8.0	2.50	0.266 ± 0.005
Nostoc muscorum G **	200	25	7.7	0.20	0.194 ± 0.003

* Variability shown is the maximum deviation from the mean in four determinations.

** Grown in large test tube cultures; other algae grown in continuous culture apparatus.

† Lyophilized cells grown under these conditions contained 48.3 % C, 6.7 % H, 9.4 % N, 4.4 % ash.

ent samples of cell suspension by washing twice in distilled water, transferring to tared crucibles in a minimum quantity of water, and drying to constant weight at 105° C. Rates of photosynthesis and respiration will be expressed in terms of Q_{O_2} with the usual dimensions of μl/hour × mg dry weight; apparent rates of photosynthesis will be cited without correction for respiration.

The growth conditions and characteristics of the cell suspensions studied are summarized in table I.

PHOTOSYNTHESIS

METHODOLOGICAL PROBLEMS: The experience of Emerson and Lewis (1) with Chroococcus and the preceding study of growth requirements of the algae used herein dictated an examination of the sodium and potassium relation in the buffers to be used. Initial experiments with *Anabaena variabilis* and *Anacystis nidulans* in 0.0125 M $KHCO_3$ plus 5 % CO_2 or in the $KHCO_3$-K_2CO_3 Warburg buffer No. 9 yielded similar results: rate of oxygen evolution decreased rapidly after a period of 20 to 30 minutes. Experiments with buffers of Na : K ratios 20 : 80, 50 : 50, and 80 : 20 gave photosynthetic rates which were steady and independent of the Na : K ratio. Subsequent studies on photosynthesis were conducted in equimolar Na- and K-buffer solutions.

Effects of centrifugation and washing were examined. Equivalent photosynthetic rates were achieved when the cells were centrifuged for one or three times and when they were unwashed or washed twice in the final buffer solution.

Three different buffer systems were examined: 1) 0.05 M phosphate at pH 5 saturated with 5 % CO_2, 2) 0.025 or 0.0125 M bicarbonate saturated with 5 % CO_2, and 3) Warburg buffer No. 9 containing 0.085 M bicarbonate plus 0.015 M carbonate. Measurements in the first two buffers require the indirect method utilizing two flasks with different liquid to gas ratios. Rates of photosynthesis observed in the three buffer systems for different cell preparations are presented in table II. Phosphate buffer at pH 5.0 supported rates only 30 to 50 % as great as those in the other buffers; it therefore was discarded for further work. Experience with Chlorella that lowered rates of photosynthesis are observed in the Warburg buffer is repeated again with *Anabaena variabilis*. However, *Anacystis nidulans* consistently shows higher rates of photosynthesis in the Warburg buffer than those observed in any other buffer system.

ASSIMILATORY QUOTIENT: Measurements of the assimilatory quotient ($AQ = -CO_2/O_2$) in bicarbonate-CO_2 buffer by the indirect method yield values at light saturation shown in table II. For *Anabaena variabilis* the AQ is typically 1.0; for *Anacystis nidulans*, about 0.9.

CONCENTRATIONS OF CARBON DIOXIDE, BICARBONATE, AND pH: Rates of photosynthesis of *Anabaena variabilis* and *Anacystis nidulans* were measured at light saturation in No. 2, 4, 6, 9, and 11 of the Warburg carbonate-bicarbonate buffer series at a total concentration 0.10 M (7, p. 178). The results are

TABLE II

PHOTOSYNTHESIS AT LIGHT SATURATION IN VARIOUS BUFFER SYSTEMS

ALGA	TEMP °C	GROWTH RATE, K	BUFFER	pH	Q_{O_2} **	AQ
Anabaena variabilis	25	0.30	0.05 M PO_4 *	5.0	74	...
			0.0125 M HCO_3 *	7.1	179	0.99
			0.025 M HCO_3 *	7.4	183	0.98
			Warburg No. 9	9.3	147	...
Anacystis nidulans	25	0.55	0.0125 M HCO_3 *	7.1	141	0.88
			Warburg No. 9	9.3	167	...
	39	1.00	0.0125 M HCO_3 *	7.1	264	0.90
			0.025 M HCO_3 *	7.4	262	0.89
			Warburg No. 9	9.3	309	...

Temperature indicated describes both growth and manometry.

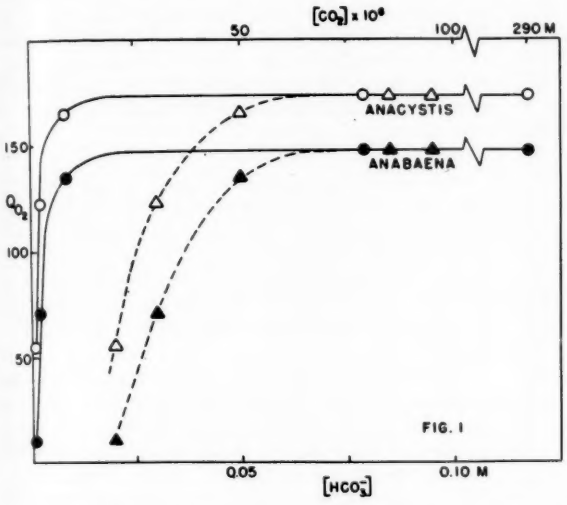
* Plus 5 % CO_2.

** Q_{O_2} has dimensions of μl O_2/hr × mg dry wt.

presented in figure 1; rate of photosynthesis is plotted in the conventional way as a function of concentration of free carbon dioxide and also as a function of bicarbonate concentration. Each point is an average of three or more closely agreeing replicate values assembled from different experiments.

In figure 2 the same data used in figure 1 are replotted as curve B describing rate of photosynthesis of *Anabaena variabilis* as a function of pH in the carbonate-bicarbonate buffer series. The curves A of figure 2 are constructed from experiments in bicarbonate-carbon dioxide buffers with the bicarbonate at 0.001 to 0.025 M in equilibrium with gas phase concentrations of 2.5, 5.0 and 10 % CO_2. In such buffers CO_2 are about the same as those observed in 0.05 M phosphate and 5 % CO_2 at pH 5 where the bicarbonate concentration is about 0.0001 M (cf table II). Comparison with the behavior of *Chlorella pyrenoidosa* is provided by curve C; rates are constant and maximum from pH 6.0 to 7.5 and are equal to that obtained in phosphate buffer at pH 4.5.

The data of figure 1 may be examined further with regard to the hoary controversy (cf 8, 10) whether free carbon dioxide or bicarbonate ion is the principal form taken up by the cells to meet their photosynthetic demand. Unfortunately, use of the Warburg buffer series is not expected to yield an unequivocal answer since there are five concomitant



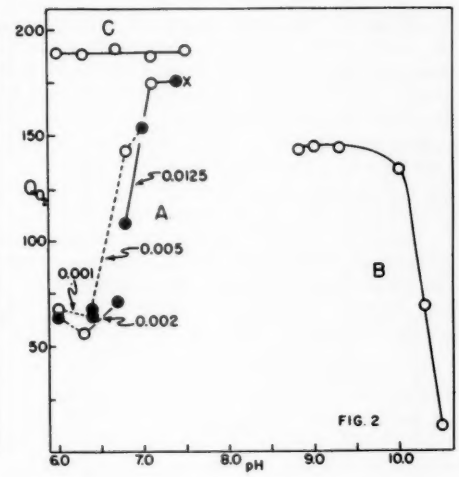


FIG. 1 (*left*). Rate of photosynthesis of *Anabaena variabilis* and *Anacystis nidulans* in Warburg buffers No. 2, 4, 6, 9, and 11 plotted as a function of carbon dioxide concentration (circles, upper scale) and as function of bicarbonate concentration (triangles, lower scale). Temperature 25° C.

FIG. 2 (*right*). Rate of photosynthesis of *Anabaena variabilis* and *Chlorella pyrenoidosa* as a function of pH. Curves A: Anabaena in bicarbonate of the molarity shown on the curves in equilibrium with 2.5 % CO_2, circle-dot; 5 % CO_2, open circle; 10 % CO_2, closed circle; X designates 0.025 M bicarbonate + 5 % CO_2. Curve B: Anabaena in the Warburg carbonate-bicarbonate buffers. Curve C: Chlorella in different concentrations of bicarbonate + 5 % CO_2.

both oxygen and carbon dioxide are exchanged with the gas phase. In any one experiment the amount of carbon dioxide bound as bicarbonate remains sensibly constant, the carbonate concentration is negligible, and therefore the carbon dioxide exchange accurately represents cell activity, so long as the bicarbonate concentration does not exceed about 0.025 M.

The overlapping curves A of figure 2 are interpreted as a single continuous curve demonstrating effects of lower pH on photosynthesis. Carbonate concentration is negligible. Overlapping rates are sustained by varying bicarbonate and carbon dioxide concentrations so that only their ratio, reflected in hydrogen ion concentration, becomes the controlling variable. It will be noted further that rates observed in 0.001 and 0.002 M bicarbonate and 2.5 to 10 % variables: the ionic strength and the concentrations of carbon dioxide and bicarbonate, carbonate, and hydrogen ion. If rates are treated as a function of free carbon dioxide only, the resulting "carbon dioxide curves" extrapolate to zero rate at zero concentration and show saturation at about 10^{-5} M equivalent to 0.03 % in the gas phase. If rates are treated as a function of bicarbonate concentration only, the resulting "bicarbonate curves" do not extrapolate to zero rate at zero concentration; further the concentrations required for saturation become far higher than those required in the bicarbonate-carbon dioxide buffers (cf fig 2). Rates might also be considered as inverse functions of hydrogen ion (cf fig 2) or carbonate ion concentrations but such treatment ignores the certain requirement of a supply factor for carbon to the cell.

Attempts were made to vary the total concentration of bicarbonate plus carbonate in buffer mixture No. 4 in the steps 0.06, 0.10, and 0.20 M. The plan of experimentation failed because of prolonged variations in rate of 0.20 M which occurred also when the lower concentrations were brought up to 0.20 M by use of chloride and sulfate as substituent ions. At 0.10 M rates were constant over the entire 90 minute period of observation; at 0.06 M the rates stabilized after about 30 minutes. At 0.06 M the observed rate was 80 % of that in 0.10 M although the bicarbonate was 60 % and the carbon dioxide 56 % as great. These observations lead to some suspicion of untoward effects of the rather high total concentration of the Warburg buffer system.

However inconclusive the results, they in no way speak against the general proposition of Rabinowitch (8, p. 891): that as a first approximation the rate of photosynthesis is a function of concentration of free carbon dioxide in the suspending medium. Such a conclusion becomes significant only because the algae studied are adapted to an alkaline environment and grow only in a limited pH range (ca 7.2 to 9.2) where the bicarbonate concentration is 10 to 1000 times greater than that of free carbon dioxide (2).

Light Intensity Curves: Rate of photosynthesis in Warburg buffer No. 9 as a function of light intensity is shown by the curves of figure 3. Light saturation of the three blue-green algae is achieved within a range of light intensities similar to that required by Chlorella. However, the shape of the light-saturation curve in the blue-greens depends upon previous culture conditions, particularly the light intensity, as previously shown for Chlorella (5). The phenomenon is demonstrated by the two curves for *Nostoc muscorum* G. Further examination of effects of temperature and light intensity of growth on cell pigment concentrations and light intensity curves of photosynthesis will be the subject of a later report.

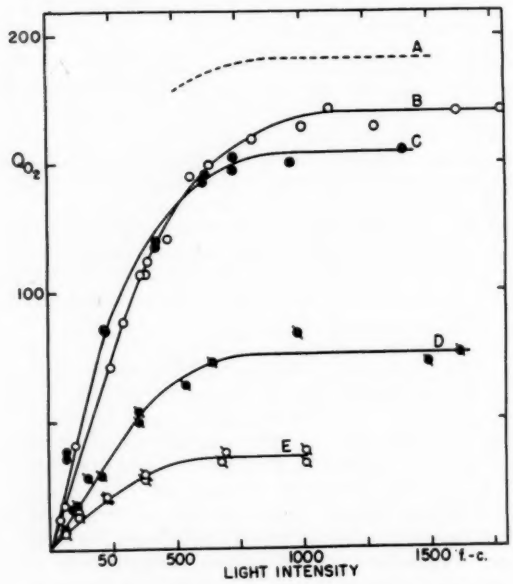


Fig. 3. Light intensity curves of photosynthesis at 25° C. A, *Chlorella pyrenoidosa*, k = 0.42. B, *Anacystis nidulans*, k = 0.55. C, *Anabaena variabilis*, k = 0.30. D, *Nostoc muscorum* G, k = 0.43. E, *Nostoc muscorum* G, k = 0.12. Values of k indicate the previous specific growth rate of the cells used.

Maximum rates of photosynthesis of *Anabaena variabilis* and *Anacystis nidulans* at 25° C are comparable to that of *Chlorella pyrenoidosa* at 25° C. At 39° C *Anacystis nidulans* has a maximum rate of photosynthesis far greater than that of *Chlorella pyrenoidosa* and greater than that of any organism yet reported except for a high-temperature strain of Chlorella (9). The photosynthetic rates of the three species substantiate the previous conclusion from growth studies (2) that the blue-green algae are not to be considered a metabolically sluggish group.

Respiration

For respiration studies two kinds of cell preparations were used. *Growing cells* were harvested from a growing culture and prepared for manometric experiments in a minimum time (35 to 45 minutes). *Starved cells* were obtained by aerobic incubation of harvested suspensions in darkness for 24 hours and at the same temperature used for growth and manometry.

The suspending fluid chosen for respiration measurements was 0.5 M phosphate buffer at pH 7.8. Equal rates were observed in 0.05 M phosphate buffer at pH 4.5, 6.0, and 9.0, in K, Na, or Na : K = 1 : 1 salts, or in original medium C. As also reported by Webster and Frenkel (11) for *Anabaena variabilis*, the pH and Na : K ratio effects so pronounced for photosynthesis are negligible for respiration in the three algae studied here.

Endogenous Respiration: Values for rates of endogenous oxygen uptake and respiratory quotients are given in table III. All three species show a rapid decrease in rate of respiration during the first 24 hours of dark starvation (growing vs starved cells) although there is only a slight decrease observed during the first hour of a manometric experiment. For *Anabaena variabilis* and *Nostoc muscorum* G the rates are within the range observed for *Chlorella pyrenoidosa* grown under similar conditions. Particularly striking, therefore, is the low rate of *Anacystis nidulans* grown at 25° C; here rate of respiration is less than 1/100 of the rate of photosynthesis. The latter characteristic is a recommendation for use in quantum yield studies. The respiratory quotients are close to unity and somewhat higher than the value 0.9 observed by Webster and Frenkel (11) for *Anabaena variabilis*.

Exogenous Respiration: Based upon past experience with other algae, starved cell suspensions were used for the examination of respiratory response to

added substrates. Any effect of added substrate is more pronounced when the endogenous rate is first reduced to a low level. In this respect starved cells of algae become similar to resting cells of bacteria. The data of table IV show that starved cell preparations of the three algae exhibit limited response to addition of glucose, fructose, and sucrose, and small responses to some of the other carbon sources tested. Experiments at pH 4.5 and 6.0 failed to enhance any of the responses shown. When added to growing cells, fructose causes no increase in respiration but does slow down the decay in rate with time.

The three algae used have been shown to be obligate photoautotrophs in the sense that none of a long list of organic compounds will support growth in the dark (2). It is considered significant, therefore, that none of the substrates examined will raise the respiratory rate of a starved cell preparation to the level of endogenous respiration shown by photosynthetically growing cells. What may be a similar case has been reported by Lewin (4); the diatom *Navicula pelliculosa* will oxidize succinate, lactose, acetate, pyruvate, and citrate but fails to grow on any of these substrates in the dark. In contrast, *Chlorella pyrenoidosa*, which can grow heterotrophically in the dark, has a rate of respiration on exogenous glucose some four times greater than that of cells harvested from a photosynthetically growing suspension.

In the case of the three blue-green algae studied here it is plausible to postulate permeability restrictions as the limitation to obligate autotrophy. In the case of *Chlamydomonas Moewusii* Lewin (3) has rejected this explanation, and perhaps correctly so, on the grounds that "compounds such as acetate, pyruvate, or succinate enter the cells and are readily oxidized in the dark." However, quantitative rather than qualitative data are necessary before an obligate character of autotrophy can be attributed to any deep-seated biochemical inadequacy. It is probable that there are organisms which would show respiratory increase to added organic substrate and utilization of such substrate to partially or completely maintain their basal endogenous metabolism, yet be prevented from growth on the substrate merely by a restricting rate of permeability. This line of reasoning leads to the likelihood that obligate autotrophy, aside from the matter of formal definition, is a matter of degree rather than an all-or-none phenomenon. The obligate character of autotrophy in the three blue-green algae, in *Navicula pelliculosa*, and in *Chlamydomonas Moewusii* may be *partial* in the sense that a part, but not all, of their carbon requirement for growth may be supplied by organic substrates.

TABLE III

ENDOGENOUS RESPIRATION

ALGA	TEMP °C	GROWTH RATE, K	CONDITION OF CELLS	Q_{O_2}	RQ
Anabaena variabilis	25	0.30	Growing	8.4	1.10
			Starved	1.7	1.00
Anacystis nidulans	25	0.55	Growing	1.6	1.00
			Starved	0.3	0.93
	39	1.00	Growing	4.7	1.10
			Starved	1.9	1.10
	39	2.50	Growing	7.5	1.05
			Starved	2.9	...
Nostoc muscorum G	25	...	Growing	4.4	...
			Starved	1.1	...

All measurements in 0.05 M phosphate at pH 7.8. Temperature indicated describes both growth and manometry. Q_{O_2} has dimensions of $\mu l\ O_2/hr \times mg$ dry weight of cells.

TABLE IV

RESPIRATORY RESPONSE TO ADDED SUBSTRATES

SUBSTRATE	ALGAE		
	ANABAENA VARIABILIS	ANACYSTIS NIDULANS	NOSTOC MUSCORUM G
	Temperature		
	25° C	39° C	25° C
	Endogenous Q_{O_2}		
	1.96 ± 0.10	28.9 ± 0.28	1.05 ± 0.06
	Exogenous Q_{O_2} *as % of control*		
Glucose	221	130	123
Fructose	195	157	142
Sucrose	166	98	150
Mannose	100	96	99
Maltose	136	...	100
Xylose	101	...	100
Galactose	98	99	...
Lactose	98	...	...
Glucose-1-PO_4	118	...	...
Succinate	130	...	123
Malate	148	...	98
Citrate	63	...	51
Acetate	104	...	...
Butyrate	42	...	...

In 0.05 M phosphate at pH 7.8; all substrates added to give 0.1 M except acetate and butyrate, 0.01 M.

DISCUSSION

The data reported provide description of characteristics of respiration and photosynthesis of three blue-green algae. As viewed in the light of more extensive knowledge of physiology of the Chlorophyceae, no startling differences are observed. In terms of the gross characteristics of elementary analysis, growth rates (2), rates of photosynthesis, and responses to light intensity and carbon dioxide concentration, the three blue-green algae are no more different from *Chlorella pyrenoidosa* than might be expected of other species of Chlorella. Only in their very limited responses to exogenous organic substrate are these algae markedly different from most of the Chlorophyceae which have been studied.

In relation to studies on photosynthesis significance lies in the presentation of *Anacystis nidulans*

as a new experimental organism with many desirable characteristics. Its cells are small ($\sim 2\ \mu$), it may be grown as easily and reliably as Chlorella, it contains phycocyanin as an auxiliary photosynthetic pigment, it can be studied over a wide range of temperatures up to 41° C, it shows no inhibitory effects of the Warburg buffers, and it has an unusually low rate of dark respiration.

Summary

Photosynthesis and respiration of three blue-green algae, *Anabaena variabilis*, *Anacystis nidulans*, and *Nostoc muscorum* G, have been studied by conventional Warburg manometry.

Rates of photosynthesis at light saturation were measured in the Warburg series of carbonate-bicarbonate buffers and in bicarbonate-carbon dioxide buffers in the pH range 6 to 10.5. Evidence was sought, but not found, that rate of photosynthesis is any simple function of bicarbonate concentration. In terms of their light intensity curves the three algae show no feature markedly different from those of Chlorella. Limited response of respiration to added organic substrates is considered related to the obligate photoautotrophic character of the three algae.

Attention is directed to *Anacystis nidulans* which has several features making it particularly useful as an experimental organism for studies on photosynthesis.

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PERSISTENT RHYTHMS OF O_2-CONSUMPTION IN POTATOES, CARROTS AND THE SEAWEED, FUCUS[1,2]

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It has been widely known for a long time that numerous plants and animals normally display overt daily rhythms of various processes and, if they live in the littoral regions of the seas, have also primary lunar cycles (tidal) of various physiological processes. In general, it has been customary to think of these as direct responses to rhythmic external factors. Similarly, the numerous instances of semi-lunar and lunar breeding cycles, common for marine animals and plants, have generally been considered to be simple responses to illumination or tides, or some combination of these.

It is gradually becoming increasingly apparent, however, that daily rhythmicity in animals possesses an important persistent aspect. The rhythmic changes persist, more or less temperature-independent as to frequency, under constant conditions of light, temperature, and humidity. It is also becoming evident that there are similarly temperature-independent persistent rhythms of primary lunar frequencies.

Beliefs as to relationships between lunar phases and human and other organismic behavior are as old as the beginnings of the human cultural heritage, but these have seldom found solid scientific support. Even when finally supported and used to predict accurately such phenomena as occurrence of plankton pulses of the Illinois River (18), the swarming of the grunion (24), or the palolo worm (17) the lack of an adequate explanation has usually left the matter in the hands of the naturalist rather than the experimentalist.

Biologists who have had occasion to concern themselves with the measurement of O_2-consumption in

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either plants or animals have almost invariably been plagued by what appeared to be a wide range of random variability in the rates of O_2-utilization even under conditions constant with respect to all factors known to influence the organisms. Gompel (16) found variations in O_2-consumption correlated with tidal times in a number of marine animals in the laboratory away from tidal changes. Brown et al (6) made analyses of the variations in rates observed in the fiddler crab, *Uca pugnax*, under constant conditions and found them capable of being resolved into a complex of rhythms. One of these correlated with the primary solar cycle of 24 hours. Another correlated with the primary lunar cycle of 12.4 hours. A third cycle of about 15 days appeared to result from the periodic reinforcement of the primary solar by the primary lunar one. A fourth cycle of about a month was observed but it was not possible with the data of the limited period of the investigation to determine decisively whether this correlated closely with the synodic month of 29.5 days or possessed another cycle length. Preliminary work with two species of snails suggested strongly that the same types of persistent rhythms of O_2-consumption were to be found in them (21).

Although the amplitudes of persistent cycles seem clearly to be a function of temperature as are the rates of biological processes in general, the frequencies of the persistent cycles appear in every case so far carefully investigated to be independent of temperature. This independence extended over as wide a range as from 6 to 26° C for the primary solar cycle and at least from 14 to 30° C for the primary lunar one in the fiddler crab (9, 10). Temperature-independent endogenous rhythms with closely similar properties have also been described for Drosophila (20) and for Avena coleoptile (1). Although the temperature-independence of the frequency of the rhythms was shown using criteria other than respiration, it seems highly likely that the frequency of the rhythms of O_2-consumption are similarly temperature-independent. This seems likely inasmuch as the rhythm frequencies correlated closely with the cosmic ones at any arbitrarily selected constant temperature used within an experiment, which was performed under conditions constant with respect to all factors now known to influence organisms.

The experiments to be reported constituted an attempt to learn whether plants possess persistent rhythms of respiratory rate comparable to those found in animals as described earlier. A brief summary of some of the results have been published (7, 8).

Among the more recent reviews of the subject of organismic rhythmicity are those of Caspers (13) and Korringa (19). The relative roles of persistently rhythmic mechanisms, on one hand, and direct induction by variations in light, temperature, O_2-tension, water and similar obvious factors, on the other, in determining the normal periodic behavior of organisms constitute an almost wholly unexplored field.

Materials and Methods

Potatoes (*Solanum tuberosum*) and carrots (*Daucus carota*) were procured from a local grocery store in Evanston, Illinois. With a large cork-borer, short cylinders about two centimeters in diameter, each bearing an eye and weighing about 5 gm were cut from the potatoes. The carrots were cut transversely into sections similarly weighing about 5 gm. The pieces of the vegetables were placed in automatic continuous recording respirometers (3) and their rates of O_2-consumption measured continuously for the 29-day period, May 12 through June 9, 1954.

An experiment with the brown alga, *Fucus*, was performed in Woods Hole, Massachusetts. Fresh Fucus was collected every 3 to 5 days. Five gm of terminal portions of thalli were placed in each of several respirometers. O_2-consumption was determined continuously for the 35-day period, July 27–August 30, inclusive, 1954.

The respirometer was of such a character that the organisms were maintained in essentially constant O_2-tension during the course of the experiments since it operates on the principle of continuous O_2-replacement. The organisms were subjected continuously to the natural variations in barometric pressure.

The respirometer consisted essentially of a 50 ml Soxhlet distilling flask as the plant-containing chamber. The chamber also contained a vessel of saturated KOH as a CO_2-absorbent. The flask was closed by a rubber stopper which was perforated by a 27-gauge hypodermic needle; attached to the top of the stopper was a collapsible, O_2-impermeable, Saran sack which was filled with O_2 (about 50 ml). The plant was maintained in air with its base immersed in a small amount of water. The flask-sack ensemble, constituting a "diver," was weighted with lead just sufficient to sink it in water and then suspended by fine wire into a constant-temperature bath. The wire activated a delicate, ink-recording, spring scale which recorded the changing weight of the submerged diver continuously upon a strip of paper drawn over a roller by a telechron clock motor. For each milliliter of O_2-consumed by the organism, the diver obviously increased in weight one gram. The walls of the bath were opaque and painted black on the inside. The apparatus was operated in a light-proof enclosure in which six 7.5-watt frosted incandescent lamps provided a constant illumination of about 1 fc at the level of the water-surface of the baths.

The pieces of potato and carrot were prepared and placed in respirometers at about 4 P.M. (all times for potato and carrot are Central Daylight, and for Fucus, Eastern Daylight) on May 10, 1954. One respirometer, possessing nine divers, was operated in conjunction with a bath maintained at 19.8° C. A piece of potato was paced in each of eight of the divers, the ninth being left as a blank control. Another respirometer, similarly possessing nine divers, had its constant temperature bath operating at 19.6° C and a carrot section was placed in each of

eight of these divers with the last one left blank as a control, as for the potatoes.

The plants were permitted to remain in the respirometers but the records for the first 31 hours were not used. Thereafter, beginning at 11 P.M., May 11, 1954, an essentially complete, 29-day record of O_2-consumption for 8 carrots and 8 potatoes was obtained.

The potatoes were all replaced by freshly cut pieces at 4 P.M. on May 14, and half of them, or four, were replaced by new ones at 11 A.M. on May 30. The O_2-reservoirs were replenished and the CO_2-absorbent was replaced at about 3- to 4-day intervals. Freshly cut pieces of carrot were substituted for the old at 5 P.M. on May 17; and thereafter only two carrots were replaced because of accidental contamination while changing CO_2-absorbent. As for the potatoes, the O_2 reservoirs were refilled and the CO_2-absorbent replaced at 3- to 4-day intervals.

Fucus was placed in each of 8 divers on the afternoon of July 26, and hourly records were taken from midnight of that day through midnight of August 30. The bath temperature was 19.6° C. Illumination at the bath surface was kept constant, about 1 fc. The O_2 and CO_2-absorbent reservoirs were refilled at each time the plants were replaced (3- to 4-day intervals).

It had been the original intention to utilize the control blank divers in order to compensate for the influences of the changes in barometric pressure upon the plant-containing respirometers but this turned out not to be feasible. When the recording system was adjusted in its sensitivity so that the full scale would permit 3 to 4 days of uninterrupted continuous recording of the O_2-consumption of the plants, the random rises and falls in barometric pressure, though qualitatively followed quite faithfully, were quantitively of smaller extent than were to be expected upon the basis of the gaseous volume and the known changes in pressure simultaneously recorded upon a precision barograph.[3] In fact, a careful correlation of 29 days of blank diver data with barometric pressure showed the divers to exhibit only about 27 % of the expected amplitude. This was finally traced to a very small freedom of movement in certain pivotal bearings of the spring scales. Since the blank divers were almost completely free from gaseous escape, the normal pressure changes produced only minute right and left excursions of the ink-writing arm about a mean. A large fraction of these excursions which were of the order of tenths of millimeters was absorbed in the pivotal bearings of the pen rather than being translated to the pen point.

This blank-diver error was confirmed also by attaching blank divers to greatly sensitized spring scales; the pivotal freedom became, thereby, smaller relative to the total barometric-pressure-change induced excursion of the pen. The blank divers under these circumstances became greatly improved barographs.

[3] Data generously provided by the U. S. Weather Bureau.

The plant-containing divers, on the other hand, appeared to behave as nearly perfect barographs during the experiments. In these instances the pen lever arm was ordinarily in constant slow movement in one direction as a result of the O_2-consumption. Here the variations in barometric pressure expressed themselves as variations in apparent rates of O_2-consumption, and the pivot of the pen arm was under continuous, essentially uniform, unidirectional tension. In figure 1 are to be found sliding averages (three consecutive hours) for 3 three-day periods of data for potatoes and carrots uncorrected for barometric pressure. Plotted directly beneath each, on the same scale, is the variation calculated for the divers on the basis of the known barometric pressure changes. It

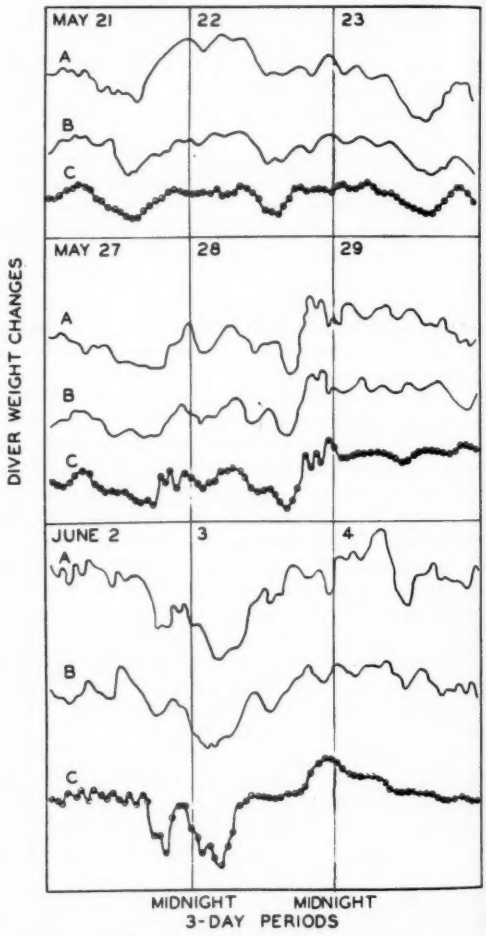

FIG. 1. A comparison for general form and amplitude, of the hourly values for three 3-day periods of A) uncorrected data for carrots, B) uncorrected data for potatoes, and C) the calculated expected change in blank divers due to the concurrent barometric pressure changes. All are plotted on the same scale.

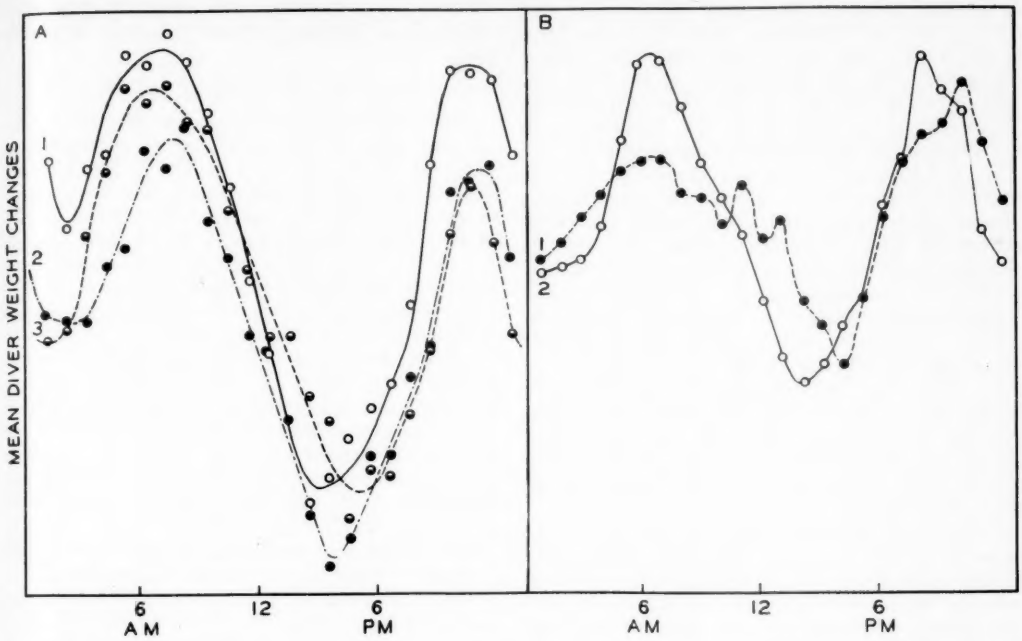

FIG. 2. A. Plotted on the same coordinates are the 29-day mean daily cycles of 1) uncorrected carrot data, 2) uncorrected potato data, and 3) the calculated variation for the respirometers due to the actual barometric pressure changes. B. The same for 29 days of data 1) for Fucus and 2) calculated barometric pressure influence.

will be noted that the general form and amplitude of the major variations are as would be expected on the basis of the divers behaving as nearly perfect barographs.

In figure 2 A are illustrated the mean daily variations of uncorrected respirometer data for the 29-day period for the carrots and the potatoes, and the calculated daily rhythm to be expected solely on the basis of the hourly barometric pressure changes during the same 29-day period. Figure 2 B illustrates the same for Fucus. All are plotted as sliding averages using three consecutive hourly values. The great similarity of not only general form and phase relationships, but also of amplitude, for the plant-containing respirometers and the calculated barometric-pressure-induced changes again gives adequate support for the assumption that the respirometers are behaving as practically perfect barographs.

In the preceding computations of barometric pressure effect, an average diver volume of 100 ml was always used and the corrections were calculated on the base value of barometric pressure, 30.00 in. Hg. Less than a 2 % error was introduced in using this arbitrarily selected value. Assays of the divers indicated a mean diver volume of about 100 ml for the 3- to 4-day respirometer runs. During this period there was a gradual volume reduction from about 110 ml to about 90 ml. Therefore, about a 10 % error was introduced through the use of this mean volume. The general drop in volume of the respirometers as the oxygen was utilized over 3 or 4 days would not contribute any rhythmic component to the frequencies being investigated. The general validity of this method is borne out by the gross good fit of the observed respirometer fluctuations and the calculated fluctuations based upon the concurrent pressure changes (fig. 1 and 2).

However, since there is a clear mean daily rhythm of barometric pressure with a range of 0.03 to 0.07 in. Hg, it would be expected that there would be a mean daily rhythm imposed upon the volume of the respirometers and hence be reflected in the data corrected for the pressure on the basis used here. At those times of day when the barometric pressure is above the average the corrections used would be too large, and for those times the pressure was below the average, the corrections would be too small. The cyclic influence of the barometric pressure would be expected to be slightly amplified, as a result of the correlated slight variation of the diver depth in the water bath and also through the operation of Henry's Law operating with respect to the water in the bottom of the respirometer chamber. A slight error is also introduced in correcting the volume as for a dry gas.

All the data used in the analyses to follow were corrected for the concurrent hourly actual changes in barometric pressure on the basis of a diver mean volume of 100 ml and a uniform pressure of 30.00 in. Hg.

The fact remains, that when one considers the form of the daily variation expected on the basis simply of the mean daily variations in barometric pressure effecting the respirometers as described, one finds in no case the form of the mean daily cycle of O_2-consumption to correspond. The rhythms appear to possess distinct species-specific components.

In the analysis of the hourly correlation between the barometric pressure rate and direction of change and the concurrent rate of O_2-consumption, the values used in the calculation of each point included indiscriminately the use of both overcorrected and undercorrected data resulting from any imposed barometric-pressure daily cycles. Hence the barometric pressure daily cycles could not have been responsible for the good correlations found.

It should also be pointed out that in setting up afresh the respirometers at three- to four-day intervals, the respirometers were closed a number of times in each 15-day or 30-day series, and in a random manner with respect to the actual pressures at the times of closure.

A diurnally rhythmic component due to the uncorrected mean daily barometric pressure cycles would be the result of overcorrection when in the cycle the pressure was above the daily mean and undercorrection when it was below the daily mean. Since there are either undercorrections or overcorrections to both rising and falling pressure changes during any period of pressure divergence from the daily mean, there would be expected to be either two or four maxima in any imposed spurious daily effect, depending upon whether the particular mean daily pressure cycle exhibited one or two diphasic variations about the mean.

Results

The rates of O_2-consumption for the potatoes during each hour of the 29-day period of observation (May 12–June 5) are found in table I. The rates are the averages for the eight pieces run concurrently. It is quite evident that the pattern of change in rate of respiration differs from day to day with maxima and minima occurring at different times. There is no overt daily rhythm. From lowest to highest, values found during the lunar period of observation involve increases of several hundred percent.

The hourly rates of O_2-consumption for the carrots for the 29-day period are found in table II. Here it is evident that injury to the carrot in preparation of slices evidently resulted in greatly accelerated initial rates of O_2-consumption which were 2 to 3 days in subsiding. The general pattern of daily changes also changes markedly from day to day in a manner comparable to that for the potatoes. From lowest to highest values over the 29-day period involved similarly an increase of several hundred percent.

A casual study of the data for the carrots and potatoes revealed that despite the fact that the mean rate of O_2-consumption of the carrot (67.33 ± 0.86 ml/kg × hr) was substantially higher than that for the potato (39.78 ± 0.64 ml/kg × hr) there was a correlation in the forms of the minor variations in rate during the whole 29-day period. A determination of the

TABLE I

Hourly values of O_2-consumption in the potato (ml./Kg.x hr.)

	A. M.												P. M.											
MAY	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
12	49	54	73	50	38	67	36	52	49	61	27	33	37	55	70	67	54	36	36	23	34	47	33	26
13	40	56	31	52	57	50	63	47	43	64	74	56	50	86	64	60	37	44	21	33	39	47	36	54
14	37	43	62	52	52	61	44	53	66	47	41	52	60	60	—	—	22	22	19	19	26	17	31	30
15	42	46	46	53	65	58	61	59	70	53	64	52	58	71	56	68	50	62	46	43	57	56	51	52
16	49	45	63	49	50	54	53	53	42	55	44	34	59	46	40	49	44	44	51	44	38	43	48	33
17	31	39	32	44	39	50	33	66	31	32	32	26	50	41	33	35	—	--	35	39	53	42	55	55
18	63	58	60	73	75	40	58	75	57	54	60	44	56	67	46	75	39	47	48	49	48	48	41	45
19	56	35	45	52	29	55	52	53	39	70	64	15	40	40	35	53	43	18	36	28	55	35	34	45
20	28	53	48	37	58	35	42	54	29	46	44	30	34	65	52	—	20	23	36	27	27	38	38	34
21	29	39	39	41	28	38	33	39	31	50	40	49	30	25	36	38	30	40	43	19	45	25	39	45
22	33	29	43	37	46	32	48	36	39	36	46	20	32	50	35	34	30	37	22	34	35	30	41	49
23	32	23	31	41	31	34	26	33	38	43	33	33	33	23	33	33	26	28	28	31	18	27	21	22
24	26	33	13	39	25	25	33	27	29	47	36	—	25	18	29	31	24	20	35	33	39	49	33	32
25	47	39	31	36	31	37	33	37	54	31	24	30	27	23	42	19	42	33	24	33	34	36	22	23
26	25	38	26	32	39	44	36	23	18	52	28	14	21	18	27	22	27	34	37	41	25	43	21	18
27	25	25	36	30	23	34	24	25	42	28	28	17	27	28	36	—	36	19	43	-24	58	34	29	35
28	21	41	32	23	36	18	38	41	25	36	34	27	46	38	36	41	38	31	46	0	100	35	55	25
29	31	40	17	37	46	23	22	43	36	13	40	48	36	21	32	34	30	46	31	16	20	1	13	19
30	25	29	18	22	31	26	32	15	21	27	—	14	41	4	48	17	38	69	51	37	43	48	61	34
31	51	16	113	75	45	26	36	45	23	38	53	52	79	77	80	42	-9	112	49	24	62	25	50	26
JUNE 1	86	71	7	118	61	53	22	41	49	22	59	42	-11	58	18	64	83	81	70	49	59	52	43	46
2	46	38	31	48	14	73	17	65	29	19	66	45	74	58	42	38	42	25	—	—	65	37	5	59
3	29	21	38	50	44	59	21	30	16	42	51	22	31	24	36	52	30	53	51	35	42	-5	17	46
4	35	43	36	36	40	45	43	41	47	69	62	22	46	44	73	39	25	46	48	38	23	70	18	54
5	--	--	—	--	51	41	42	36	31	40	35	43	24	25	18	31	—	21	20	48	44	37	28	38
6	27	51	36	45	48	29	45	33	58	38	29	—	23	20	49	37	34	32	51	33	60	26	34	35
7	17	31	34	53	47	33	27	41	31	60	28	44	36	33	46	31	27	43	45	29	33	68	26	23
8	31	40	29	50	40	41	17	51	17	48	17	41	20	33	47	40	38	5	0	23	35	33	47	19
9	19	41	15	42	31	44	28	46	5	48	12	82	53	21	21	9	19	41	25	45	51	33	27	20

TABLE II

O_2-consumption in the carrot (ml./Kg.x hr.)

	A. M.												P. M.											
MAY	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
12	122	87	66	52	47	107	69	90	93	70	93	69	104	77	64	59	74	64	95	72	96	61	61	77
13	64	73	74	72	99	64	73	57	82	70	63	64	47	77	—	—	60	54	54	58	61	73	52	67
14	86	54	40	80	—	—	47	67	—	—	42	30	—	—	—	—	47	38	21	47	22	51	29	49
15	71	49	64	46	83	42	59	62	64	59	53	44	56	44	67	40	50	46	40	50	55	39	53	67
16	42	59	67	53	64	56	50	75	39	63	40	52	57	54	59	57	56	56	63	56	60	39	53	66
17	54	39	36	74	39	66	47	50	39	45	47	50	66	37	40	—	—	91	112	100	125	123	119	119
18	160	125	122	150	150	95	125	129	114	113	130	102	127	125	104	107	107	101	99	115	93	99	86	95
19	107	103	93	93	92	125	82	88	94	104	101	79	94	90	105	87	84	69	75	57	89	81	94	87
20	83	77	98	83	91	97	91	95	85	90	84	84	85	69	83	87	57	55	86	74	64	81	70	78
21	70	71	78	76	61	68	60	81	56	70	63	79	56	74	79	59	72	83	74	79	95	80	86	103
22	92	85	83	83	98	89	107	82	88	93	94	61	87	85	80	87	63	68	78	65	69	61	81	90
23	67	53	69	79	66	57	58	69	70	64	70	60	50	50	47	47	51	66	48	70	49	63	64	60
24	47	57	54	57	50	61	56	53	41	75	—	—	58	63	86	75	83	81	85	95	103	104	83	99
25	86	101	92	82	98	85	106	81	99	83	80	96	73	80	74	60	97	86	93	103	89	66	65	76
26	80	107	93	118	59	84	74	83	76	46	49	78	65	66	71	73	82	74	71	70	56	68	55	55
27	61	82	58	73	78	37	58	48	74	63	64	55	52	—	—	—	70	51	64	9	75	78	60	66
28	61	65	59	45	72	53	70	66	53	66	78	40	77	79	60	62	52	45	75	40	131	24	72	38
29	57	76	63	77	54	76	51	73	71	56	64	73	69	58	53	49	50	67	36	46	46	36	44	47
30	49	49	46	48	44	46	51	32	48	51	65	—	33	29	51	27	46	49	56	53	62	55	62	63
31	65	51	44	82	35	58	39	51	62	45	55	76	34	90	107	60	36	110	80	54	83	94	61	39
JUNE 1	68	53	57	120	74	86	68	66	69	68	67	77	53	108	92	92	92	94	104	83	95	84	84	69
2	66	83	50	84	31	101	47	91	60	50	89	50	73	68	69	75	58	31	78	55	68	53	13	86
3	50	41	51	49	44	54	36	28	21	31	68	30	70	39	49	68	78	58	57	57	31	40	47	66
4	49	68	70	61	56	71	97	75	82	79	3	64	54	60	73	52	88	48	66	75	41	63	58	101
5	58	46	91	71	74	81	77	76	79	62	74	61	54	60	53	46	44	48	60	46	60	71	37	59
6	39	78	42	73	49	51	58	68	51	77	65	—	31	32	61	48	52	51	59	55	80	41	51	65
7	36	54	40	54	61	64	34	60	60	70	37	65	32	33	53	55	43	63	60	71	63	74	71	66
8	64	56	53	66	59	64	57	50	48	80	62	60	45	62	49	52	63	61	46	45	64	29	69	63
9	41	56	50	81	56	66	36	60	27	54	37	83	29	20	42	49	43	67	33	48	64	44	21	40

coefficient of correlation for the hourly data of the two yielded a value of 0.37 ± 0.033. Since the coefficient is more than 11 times its standard error the reality of a correlation cannot be questioned. It should be emphasized here, that the carrots and potatoes were being run in different constant temperature baths, at slightly different temperatures, and at different locations.

The hourly rates of O_2-consumption for the alga, Fucus, for the 35-day period, July 27–Aug. 30, are found in table III. As with the other species there appear to be relatively large, apparently random,

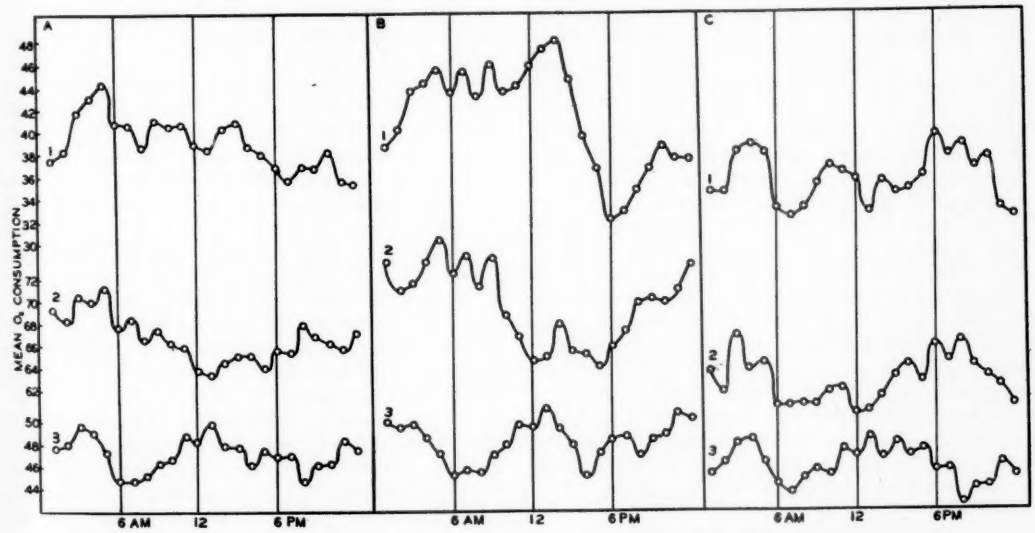

FIG. 3. A. The average 29-day diurnal cycles of 1) potato, 2) carrot, and 3) Fucus. B. Average 15-day daily cycles for the 1) potato, 2) carrot, and 3) Fucus, over a period extending from first to third quarter of the moon. C. Same as B but for a period extending from third to first quarter of the moon.

TABLE III

O_2-consumption in the Fucus (ml./Kg.x hr.)

		A. M.												P. M.									
JULY		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
	27	35	55	38	45	39	42	35	41	52	60	37	31	50	25	37	53	28	38	32	19	50	38	32	40
	28	44	40	32	37	37	20	52	24	48	45	38	37	43	41	35	15	27	38	39	53	29	13	40	53
	29	54	43	29	38	45	45	48	37	31	57	13	24	39	54	38	51	42	50	47	42	50	53	45	53
	30	37	67	63	49	52	57	43	50	—	—	—	53	57	45	43	48	38	77	41	46	57	52	55	56
	31	48	60	67	65	63	56	72	55	62	50	70	73	62	64	64	57	82	57	52	67	59	55	55	75
AUGUST	1	72	37	72	70	55	69	50	53	65	52	55	79	42	45	57	35	72	41	46	70	51	42	65	53
	2	58	43	57	55	50	56	58	48	39	43	53	5	53	42	48	57	49	47	38	48	48	37	49	45
	3	45	60	50	35	36	47	36	60	53	—	37	48	40	52	44	39	45	38	43	35	36	38	28	41
	4	30	35	31	42	39	35	25	37	30	—	50	50	50	46	59	41	50	35	45	45	30	55	43	39
	5	55	43	44	55	38	40	58	39	62	45	47	65	55	69	45	71	5	75	58	35	53	37	68	34
	6	52	42	33	45	43	53	36	40	44	41	33	56	26	58	—	43	43	48	32	56	45	50	40	52
	7	43	50	57	48	48	41	40	42	52	48	57	60	61	47	35	35	52	40	55	43	44	67	41	57
	8	50	48	48	58	41	41	32	43	53	41	48	55	41	43	48	45	53	59	38	45	51	50	60	49
	9	45	58	53	57	53	48	43	50	—	—	53	41	41	89	62	13	45	47	48	48	35	27	26	—
	10	—	—	50	23	33	45	27	42	28	30	43	35	25	32	39	30	28	33	30	40	20	44	25	50
	11	29	32	44	34	33	26	35	48	32	43	35	45	35	—	34	25	31	55	44	43	53	70	64	70
	12	70	72	80	76	57	60	61	63	62	89	105	96	95	90	84	61	84	83	81	69	79	79	81	77
	13	92	98	90	73	79	67	80	83	—	—	62	60	50	48	49	43	40	46	35	45	30	51	45	48
	14	38	40	48	36	41	40	33	52	35	43	38	37	37	32	42	34	30	35	44	40	35	55	38	40
	15	33	46	35	40	44	37	38	42	37	37	44	45	39	45	38	45	30	27	25	51	17	50	44	32
	16	29	48	41	15	57	32	44	39	42	30	14	32	31	69	39	30	54	29	29	35	35	46	49	28
	17	6	25	22	35	13	18	27	27	—	—	60	70	60	67	52	57	55	63	42	52	47	57	51	50
	18	48	40	60	47	51	44	56	53	43	52	53	54	53	38	55	55	55	55	50	55	35	56	47	46
	19	48	45	43	49	62	49	43	53	58	48	48	52	63	57	50	60	70	50	60	40	—	57	55	63
	20	63	52	77	65	42	70	61	57	60	—	—	75	62	45	57	62	48	62	48	42	60	50	43	51
	21	35	44	40	55	45	32	51	29	53	37	53	62	45	38	40	42	38	42	43	70	27	37	38	41
	22	41	33	28	77	57	12	35	28	42	45	42	35	44	59	35	50	43	38	28	45	33	48	48	28
	23	33	45	40	38	50	27	48	31	29	—	—	60	76	69	59	64	60	57	50	49	57	53	48	68
	24	56	43	49	62	52	53	42	58	40	60	43	62	53	55	48	57	56	42	45	47	46	57	53	49
	25	45	64	48	43	49	41	52	57	53	48	53	57	42	61	37	43	51	60	42	53	56	38	52	49
	26	63	57	55	61	60	45	39	55	50	61	50	40	67	45	76	55	41	76	59	35	46	28	48	46
	27	37	41	48	50	32	56	19	41	46	51	32	43	39	29	24	38	30	49	50	48	27	35	48	40
	28	41	32	48	43	35	44	36	56	32	36	60	33	46	40	42	36	45	28	31	49	25	28	38	38
	29	25	37	43	40	53	44	42	41	51	41	34	35	51	43	35	60	53	40	60	41	46	37	46	51
	30	44	54	61	53	49	56	53	34	59	57	29	41	44	—	52	41	44	59	40	31	47	43	55	38

fluctuations in rate through days and also from one day to the next.

The mean rate of O_2-consumption was found to be 48.29 ± 0.59 ml/kg × hr. A study of possible correlation of the hourly changes with those for the potato revealed there to be no significant one (0.0527 ± 0.039) despite the fact that the hourly correlations were made in such a manner as to bring as closely into alignment as possible any existing solar and lunar cycles for the two series of data, by synchronizing not only times of day but phases of the moon.

The random character of the day-by-day variations in O_2-consumption was clearly evident when one attempted to correlate the hourly rates of O_2-consumption of the carrot and the potato, but now after dislocating the two series of data by one day (i.e., the hourly data of day n for the carrot was correlated with the hourly data of day n + 1 for the potato). Despite the similarity of the phases of the solar cycle, and the close similarity (51 minutes off) of any lunar cycle a determination of a coefficient of correlation over the 29-day period yielded a value of -0.016 ± 0.04, obviously indicating no measurable correlation.

Analysis of the Results

To determine whether rhythms of any frequencies of known external physical cycles were present in the potatoes, carrots, and Fucus, the data were first analyzed in such a manner that any existing primary solar cycle of O_2-consumption would be essentially stripped of any complicating primary lunar one. This was done through the use of the whole 29 or 30 days of data. In such a synodic monthly period every phase of a primary lunar cycle would have just scanned once the hours of a primary solar cycle. Hence, for example, the 1 A.M. hour over such a period would have within it every phase of the 24.8-hour cycle, as would have every other hour of the day. In figure 3 A are plotted the average rates of O_2-consumption for every hour of the day for the monthly period, for potatoes, carrots, and Fucus. There is a distinct daily rhythm of O_2-consumption in the potato with a maximum about 5 A.M., a general tendency to remain high through the day with secondary maxima in the late morning, and middle afternoon, and a tendency for a drop after 3 P.M. with a small maximum in the late evening.

A comparable analysis of the data obtained for the carrot similarly reveals an unequivocal daily rhythm. In this case, the maximum for the day similarly occurs about 5 A.M., and there is a minimum just after noon. There is a steady but irregular increase in rate from the noon low to the next morning high.

Fucus similarly exhibits a persistent daily rhythm with conspicuous maxima at 3 A.M. and 1 P.M., but

with lesser ones at 6 and 11 P.M. Broad minima occur about 7 A.M. and 8 P.M.

In figure 3 B are found the daily rhythms of the three plants calculated on the basis of a 15-day period, from first quarter to third quarter (centering on full moon) of the moon. Here, each half of the solar day is scanned by a different portion of a lunar-day cycle—a zenith or a nadir tide. In figure 3 C are comparable daily rhythms for the three plants for the 15-day period from third quarter to first quarter of the moon (centering on new moon).

In all three species it is evident that the form of the daily cycle differs between the two semi-lunar periods. The predominant difference for all three is that the mean daily rate of O_2-consumption is lower in the fortnight centered on new moon than in that centered on full moon. Also, the afternoon or early evening values, or both, tend to be relatively higher, compared to other times of day, in the 15-day period straddling new moon, than for that straddling full moon.

In order to attempt to understand further the significances of these variations, or at least to learn their correlation with external cosmic events, figure 4 was prepared. In this figure are found for the three plants the relationship between phase of moon and 1) average daily rate of O_2-consumption (individual daily means) 2) average rate of O_2-consumption for two 3-hour periods, 5–6–7 A.M. and 5–6–7 P.M. (sliding averages of three days). All the plants exhibited considerable fluctuations in rate over the month of observation. The potato in both the daily averages and the averages for the two selected periods of the day tended to show general maxima which were, except for the 6 A.M. period, at or near the times of full and new moon. The time of new moon was correlated with a relatively low rate at 6 A.M. This difference was obviously the basis of the change in form of the average daily variation over full moon, on the one hand, and new moon on the other. From lowest to highest values for the month, even for these sliding averages which obscure extreme variations, was an increase of more than 100 %.

The values for the carrot correlated to some extent with those of the potato. Full moon, however, was correlated with a relatively low value. The high values just following this time are probably, in part, artifacts of an injury-induced increase in O_2-consumption. Although such injury probably resulted in higher values than otherwise for the values for May 17–May 21, inclusive, the data suggest that a lesser maximum would probably have occurred at about the same time had not the carrots just then been replaced with freshly-cut pieces. The carrot showed another distinct difference from the potato in having a maximum near the time of third quarter of the moon, a time when respiration in the potato was near minimal for the 29-day period. But, as for the potato, the carrot showed a 6 A.M. minimum about the time of new moon when on the same day a relatively high rate was found for 6 P.M. This latter would

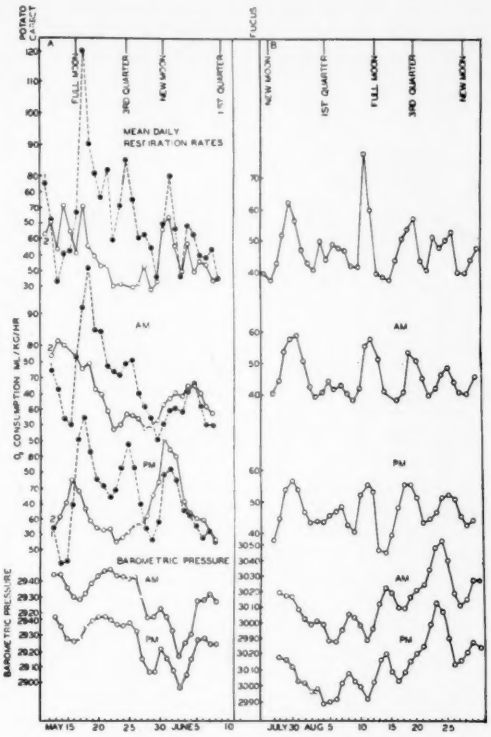

FIG. 4. A. The variation in mean daily rates of O_2-consumption for 1) carrot and 2) potato. The mean rates for the 5–6–7 A.M. period and the mean rates for the 5–6–7 P.M. period, for the carrots and potatoes and the variations in the 5–6–7 A.M. and 5–6–7 P.M. values of barometric pressure are also shown. B. The comparable relationships for Fucus. Except for the mean daily rates, all are 3-day sliding averages.

clearly account for the differences in the general forms of the two fortnightly average daily variations for the carrot.

Just as the carrot differed from the potato in the rate for the two periods of the day (6 A.M. and 6 P.M.) so did it also differ in the variation in mean daily rates. Here the carrot tended toward high rates not only about the time of full and new moon, but also about the time of third quarter of the moon.

Compared with the potato and carrot, the form of the daily variation in respiration of Fucus differed less between the two fortnights. Both the variations in the daily mean values and the mean values for the two periods of the day showed a periodic fluctuation with maxima occurring at 6- or 7-day intervals and with amplitude of variation from low to high values, of the order of 40 %.

Plotted also in figure 4 are sliding averages of the values for barometric pressure for two 3-hour periods, 5–6–7 A.M. and 5–6–7 P.M. For the potato there is suggested a rough inverse correlation with barometric

pressure. The carrot tends to show maxima correlated with both relatively high and relatively low values of mean barometric pressure. For Fucus there seems to be, as for the potato, an approximate inverse correlation between mean barometric pressure and mean rate of O_2-consumption.

To determine next the character of any existing primary lunar rhythm, it was necessary to render random the persisting daily cycle. To do this, the 24 hourly values for the second day of the 29-day series for each plant were placed directly under those of the first day. The data for the third day were placed below those of the second, but now every value was shifted one hour to the left so that, for example, the 2 P.M. value for the third day lay beneath the 1 P.M. value for the second. The 1 A.M. value was then displaced to the opposite end of the line under the 12 P.M. value for the second day. The data for the fourth, fifth, and sixth days were similarly each shifted an hour to the left relative to the lines directly above them, and the early morning hours which no longer lay beneath the values for the first day were for each day removed as a block to the hight hand end of the same line to complete a 24-hour row. The remaining days of data of the 29-day series were used in the same manner to move successive days of data backwards over earlier days at the rate of five hours in each six days or, in other words, at an average rate of 50 minutes a day. It was now clearly evident that a primary solar cycle had just scanned once the 24 vertical columns of data, and hence was neutralized. On the other hand, any cycles of primary lunar frequency were now closely synchronized in the successive days of data. In figure 5 A are shown the primary lunar variations in O_2-consumption for the potato and carrots and for Fucus, studied 3 months later. The values in figure 5 are sliding averages of 3 hours. The approximate times of lunar zenith and nadir relative to the variations found are indicated in the figure legend.

It will be noted that for all three species the lowest rate of respiration for the lunar day preceded lunar zenith by 1 to 3 hours and all showed a lesser minimum 1 to 3 hours before lunar nadir. It is evident that for respiration in these organisms lunar zenith and nadir are not correlated with quantitatively similar variations. The pattern of the lunar-day (24.8-hour) cycle of O_2-consumption for the carrot resembles in a general manner that of the potato and Fucus except for a greater tendency to exhibit what might be referred to as quarter lunar-day cycles (i.e., with minima between the primary ones correlated with zenith and nadir).

In view of the possibility that the rhythms of respiration which were here observed under "constant" laboratory conditions might be directly induced by rhythmic variations of the same frequency of some external factor or factors other than light, temperature or humidity, an analysis was made of the relationship between the hourly rates and direction of barometric pressure change and the rate of respiration. The coefficients of correlation for the hourly values for these two phenomena for 29 days of data were determined for the three plants. All three plants showed significant negative coefficients. The value for the potato was -0.585 ± 0.035; for the carrot it was -0.176 ± 0.037; and for Fucus it was -0.306 ± 0.034. In other words, in a general way the more rapid the rate of barometric pressure rise, the lower the rate of respiration and the more rapid the rate of fall, the higher the rate of respiration. Just as there were species differences in the forms of the daily cycles, so were there substantial differences in these correlations.

The relationship between rate and direction of barometric pressure change and the concurrent rate of respiration are shown in figure 6 A. The potato shows a reduction in O_2-consumption in proportion to the rate of pressure rise and an increase in proportion to the rate of pressure fall. An increase or decrease of about 10 % for each 0.01 in. Hg/hr change is seen.

The same general form of relationship is found for Fucus, except that there is only about a 5 to 6 % change in O_2-consumption for each 0.01 in. Hg/hr change in barometric pressure.

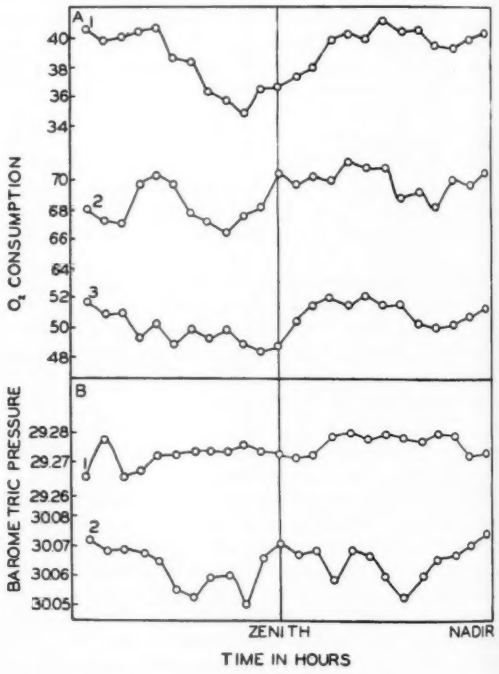

FIG. 5. A. The mean 29-day primary lunar cycle in respiration of 1) potato, 2) carrot, and 3) the 30-day primary lunar for Fucus. B. The concurrent mean lunar-day barometric pressure cycle for the 1) 29-day period of the potato and carrot study and 2) the 30-day period of the Fucus study. Lunar zenith lies above the E of zenith and lunar nadir above the I of nadir.

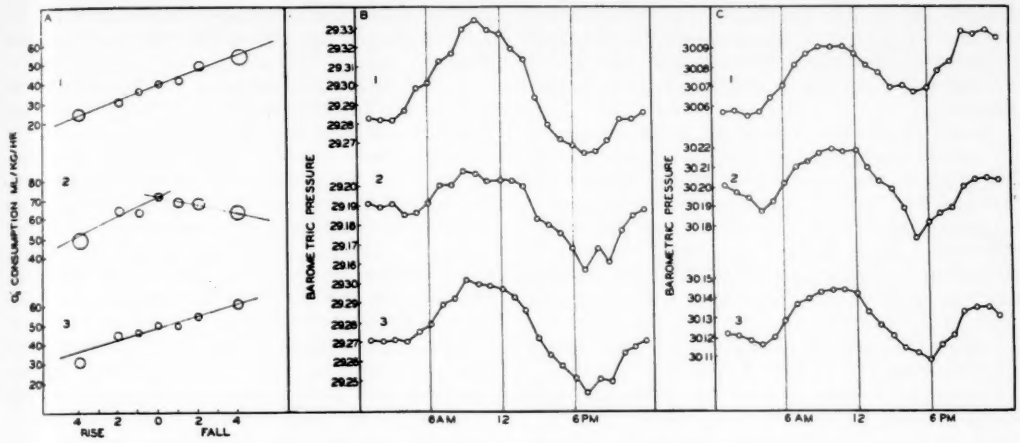

FIG. 6. A. The relationship observed between the rate of rise or fall of barometric pressure in 0.01 in. Hg/hr and the mean rate of respiration for 1) potato, 2) carrot and 3) Fucus. The pressure rate changes, 0.03, 0.04, and 0.05, have been combined because of the few data. The size of the circle denotes the standard error of the means. B. The mean barometric pressure cycles for the same periods for which the mean daily rhythms were determined for the carrot and potato. 1) The fortnight centered on full moon, 2) the fortnight centered on new moon, and 3) the 29-day period. C. The comparable mean daily cycles for the period study of Fucus.

The carrot which, it will be recalled, exhibited the lowest general correlation with barometric-pressure change, shows a qualitative difference from the other two plants. The carrot displayed a reduction in respiration rate in response to both rise and fall in barometric pressure. To rise in barometric pressure there was a reduction in O_2-consumption of about 7–8 % for each 0.01 in. Hg/hr. On the other hand, there was a drop of about 3 % for each 0.01 in. Hg/hr fall in pressure. It is interesting at this point to recall that the carrot tended to show maxima in the various earlier plots of variations in O_2-consumption with time, not only nearly synchronously with potato maxima but also often at the times of potato minima.

Very similar daily and primary lunar rhythms and correlations with barometric pressure change have been found for a vertebrate, *Triturus*, and for two species of fiddler crabs among the invertebrate animals (unpublished work). Furthermore, the fiddler crabs which were studied for two months continuously exhibited not only the same kind of general form shift in the daily cycle in going from the first fortnight straddling full moon to a second fortnight straddling new moon, but repeated the difference the second month.

That the daily and lunar rhythms with their monthly variation is not an artifact due to improperly corrected respirometer data is found by work on the oyster (4), whose activity was automatically recorded for 45 continuous days under constant conditions by simply measuring the mechanical opening and closing of the shells through threads attached to them and to ink writing levers recording on a continuous strip of paper. The daily, lunar, and monthly cycles were of the same character as for the plants. It resembled, in considerable detail, including the hourly correlation with barometric pressure change, the relationships found for Fucus.

It is, therefore, evident that at least one component in the persistent daily rhythmicity in O_2-consumption is a direct induction by some external factor. This fact was supported by correlating the hourly rates of O_2-consumption for a month with the hourly changes in O_2-consumption displaced by exactly 24 hours. When this was done for Fucus, the value of the coefficient of correlation was only $+0.115 \pm 0.038$. A comparable study for the potato yielded a value of 0.217 ± 0.038. These values are of the general order of size as found for correlating pressure changes, day n, with pressure changes, day $n+1$, (0.176 ± 0.036) on day $n+2$, (0.136 ± 0.037). It will be recalled that carrot and potato respiration failed entirely to show any significant correlation when the data for day n for one was correlated with day $n+1$ for the other (-0.016 ± 0.04). Similar lack of hourly correlation was found between the values of Fucus and the potato studied at different times, even when the data were synchronized with respect to time of day and phase of moon (0.053 ± 0.039). These last lacks even of suggestions of a correlation again points to the species differences in the cycles.

The 15-day and 29-day rhythms of barometric pressure for the periods of study of the carrot and potato, on the one hand, and for the period for Fucus, on the other, are illustrated in figures 6 B and 6 C, respectively. It is obvious that the forms of the cycles are slightly different for the two locations and times of year. It is apparent here, as is well known, that there is a daily cycle of signs and magnitude of rates of change of barometric pressure which are sta-

tistically almost as precise as are the daily variations in illumination. It is also known, however, that there are larger random, hour by hour, and day to day, variations than are true for visible light under natural conditions.

It will be recalled from figure 4 that the day to day variations in mean O_2-consumption appear to be, in general, inversely correlated with the slow gradual rises and falls in mean daily barometric pressure in a comparable manner to that of the hour-by-hour responses. However, on an hour-by-hour basis throughout the experimental periods, a correlation with the actual barometric pressure was either small or could not be seen. For the potato a correlation of -0.024 ± 0.038 was obtained; for the carrot, $+0.162 \pm 0.038$; and for Fucus $+0.005 \pm 0.039$.

It has been known (13a) that there are barometric pressure rhythms of primary lunar frequency. These are of much lower amplitude than the primary solar; on the average of the order of 0.004 in. Hg, and only capable of rough approximation as to form and frequency even with 29 or 30 days of hourly barometric-pressure data. In figure 5 B is plotted the primary lunar variation in barometric pressure, for the period May 12 through June 9 at Evanston, Illinois, and also the comparable variation for the period July 22 through August 20 at Woods Hole, Massachusetts. The primary lunar variation in respiration of Fucus at Woods Hole might be in some measure accounted for in terms of direct induction by some external factor correlated with barometric pressure since maximum rates of barometric rise (cf. respiration reduction) occur shortly before lunar zenith and lunar nadir and maximum rates of fall (cf. respiration increase) tend to occur 5 to 6 hours after lunar zenith and nadir respectively. Any primary lunar cycle of pressure was much lower in amplitude for the month, May 12 to June 9, in Evanston, Illinois. Furthermore, the general form of the mean lunar-day variation which is present has phase relationships which differ greatly from those obtained at Woods Hole at the later dates. Hence, this cannot account for the carrots and potatoes both having mean primary lunar cycles, essentially in the same phase relationships to the actual lunar cycles as with Fucus, and, in the case of the potato, having even greater amplitude.

Qualitatively, the form of the 29-day daily variation in respiratory rate of Fucus is explained fairly well in terms of the hourly correlation of rate with concurrent barometric pressure change. The early morning barometric pressure drop is correlated with an early morning maximum, the middle A.M. rise in barometric pressure is associated with a minimum in respiratory rate, and the early afternoon rapid fall, with a peak in respiration. The early evening rise in barometric pressure is correlated with a second minimum in respiration. It is very important to recall from the earlier discussion that this is not the mean cycle form expected from any insufficiency of correction of the respirometer data for barometric pressure changes, although some of the minor fluctuations in the general form of the cycle may be so explained.

In the carrot it is apparent by inspection of the plots of the daily rhythms of barometric pressure and respiration, that, as expected from the relationship of respiratory rate to rate of pressure change, the highest rates of respiration occur during the hours of relatively little pressure change, particularly during the early morning and early evening hours.

For the potato, it would appear that the morning rise in pressure is correlated with a reduction in respiration and that the steep afternoon drop is correlated with one of the daily peak rates.

Also, the general form differences between the first and second fortnights in all three plants clearly reflect the differences in the forms of the pressure daily rhythms for the same periods, when interpreted in terms of the discovered relationships between rates of pressure change and respiration.

A full explanation of the form of the respiration cycles would appear to require the interpolation of a more purely biological component, perhaps, e.g., including periods of compensatory accelerated respiration following periods of directly induced reduction in rate. Also, whatever external factor operative, the organism may be capable of exhibiting accommodation, adaptation, or other relatively rapid regulatory reaction.

In view of the fact that a purely endogenous 24-hour cycle can occur in an animal, even a lower, cold-blooded one (11), it seems quite possible that the plant may similarly not be entirely passive in its response to the external cyclic factors. Some of the divergence from the form of response to be predicted from the pressure changes may be due to such endogenous cyclic contribution. The phenomenon of photoperiodicity as it is known, of wide importance through both the plant and animal kingdoms, implies the possession of a relatively accurate and temperature-independent physiological time-sense, since in this phenomenon, there is, within rather broad limits, a relative independence of the illumination intensity and often even of simultaneous temperature.

Also, the highly precise solar and lunar rhythmicity that synchronizes gamete liberation in certain of the marine algae (e.g., *Dictyota*) even when directly shielded from normal full daylight or moonlight would appear to require time-measuring mechanisms of considerable accuracy to serve as insurance mechanisms for unusual meteorological conditions. There is some evidence that diatoms do possess such lunar biological clocks (14). These clocks could normally maintain the frequency of the cosmic cycles by such a mechanism as is described in this report.

This research gives little suggestion as to what external rhythmic forces are involved. Virtually every known physical factor of the type that could be operative here exhibits some degree of correlation with barometric pressure.

It is quite reasonable to postulate that the fre-

quency of these cycles of O_2-consumption of daily and lunar frequency in the potato, carrot and Fucus are independent of random temperature variations during the month. This has been shown to be true for rhythms of similar forms and/or frequencies, those of activity in the oyster (4), of color change in the fiddler crab (10), and for rhythms of emergence in Drosophila (12, 20). Despite the fact that the carrots and potatoes had been kept at room temperature and also in a refrigerator at different times over a period of days, all seemed when placed in constant conditions of light, temperature, and humidity at the arbitrarily selected temperatures of 19.6 and 19.8° C to possess cycles of the same frequencies and a rhythm having not only the same frequency as the primary lunar cycles but even the same phase relations. It must be presumed that the frequencies would have been the same whether the temperatures during the experiments had been held, for example at 15 or at 25° C. The form and amplitude, however, may well be influenced by temperature.

The absolute rates of O_2-consumption, on the other hand, undoubtedly are a function of the temperature with a Q_{10} in the expected range for biological processes. It seems likely that the differences in form of the daily cycles of the several plants can be interpreted in some measure in terms of their specific responses to some fluctuating physical factor or factors which, in turn, are correlated with barometric pressure. It is possible that pressure changes themselves may serve as stimuli.

It is tempting to speculate that the organisms are responding primarily to some factor other than pressure. Suggesting this is the similarity of the lunar rhythms of the three plants despite considerable differences in the form, amplitude, and phase relationships of the simultaneous mean lunar day cycles. The individual daily patterns of cosmic-ray bombardment of the earth (22) show the same more or less irregular gradual changes of form from day to day, and these appear to be capable similarly of resolution into cycles of primary solar length (2) and in addition into lower amplitude cycles which could well be of primary lunar frequency (23). The cosmic-ray variation at ground level is in some degree influenced by the barometric pressure variations and hence have forms of daily patterns which are in part correlated with the barometric pressure. Suggestive of a possible role of cosmic-ray showers, whose intensity would be correlated with barometric pressure, and yet independently possesses a clear daily rhythm (15) is the demonstration of a measurable physiological response of an organism to experimental variation in intensity of cosmic-ray induced showers (5).

Summary

1. The O_2-consumption of sections of carrot, pieces of potatoes containing eyes, and tips of the thallus of *Fucus* was measured continuously over 29-day or lunar month under constant conditions. Slow variations ranging over as much as 3- to 5-fold differences were observed during this period, with the pattern of variation changing from day to day.

2. The mean daily rate of respiration of the potato showed a 15-day cycle with maxima near the times of full moon and new moon. The carrot exhibited a minimum about the time of full moon and maxima at about third quarter and new moon. Fucus displayed cyclic variations in rate of respiration with maxima at 6- to 7-day intervals.

3. Both the carrot and the potato, while exhibiting maxima in mean daily rates of O_2-consumption at the time of new moon, showed minimum rates about this time of the month for the 5–6–7 P.M. period.

4. Through the use of 29 complete days of data it was possible to obtain the form of the mean daily cycle of O_2-consumption, since in 29 days all phases of a primary lunar cycle are present at each hour of the day.

5. The average daily pattern for the 29-day period for the potato was found to show a maximum at 4 to 5 A.M. and lesser maxima at 10 A.M., 3 P.M. and 10 P.M., highest rates were observed in the morning hours and the rate gradually decreased through the day to the lowest ones in the evening.

6. The average daily pattern for the 29-day period for the carrot was found to show a conspicuous maximum at 4 to 5 A.M. and a minimum just after noon.

7. Fucus exhibited maxima at 3 to 4 A.M. and 1, 6, and 11 P.M. with principal minima at 7 A.M. and 8 P.M.

8. In all three species the mean daily rate and form of the daily variation for a fifteen-day period straddling full moon differed from that for the fifteen-day period straddling new moon, and in the same qualitative manner.

9. An analysis of the data performed in such a manner as to determine the character of any existing persistent primary lunar cycle of O_2-consumption revealed that all three plants possessed such cycles, and the cycles were similar in form and phase relationships. All showed a primary minimum one to three hours before lunar zenith, and a lesser minimum 1 to 3 hours before lunar nadir.

10. A significant correlation was found between the hourly rates of respiration and the concurrent rate of change in barometric pressure for all three plants.

11. The rate of O_2-consumption of the potato was decreased about 10 % for each 0.01 in. Hg/hr rise in barometric pressure, and increased by about the same amount for each 0.01 in. Hg/hr fall.

12. Fucus respiration was decreased or increased about 5 to 6 % for each rise or fall, respectively, in barometric pressure of 0.01 in. Hg/hr.

13. The respiration of the carrot was decreased by both rises and falls in barometric pressure, decreasing about 7 to 8 % for each 0.01 in. Hg/hr rise and decreasing about 3 % for each 0.01 in. Hg/hr fall.

14. The forms of the daily and lunar rhythms of respiration are discussed in terms of these correla-

tions and the measured daily and lunar-day barometric pressure cycles.

15. There is also some suggestion of a correlation between the mean daily rate of respiration and the gradual rising or falling of barometric pressure over hours or days.

16. There is a brief discussion of what might be the nature of the operating external factors, of the possibility of an endogenous rhythmic component in the plants, and of possible functional significance of these rhythms to plants.

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BRIEF PAPER

THE PHOSPHORUS-IRON RELATIONSHIP IN GENETICAL CHLOROSIS[1]

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Variegation of leaves is found widely distributed in the genera of the plant kingdom. Many forms are of great intrinsic beauty and are therefore a common feature in estates and gardens. The chlorotic areas usually appear as pale edges of varying width and regularity around and overlying an otherwise apparently normal green leaf, but very often whole leafy branches may appear colourless while others may grow out normally green, indicating that the variegated plant is in fact a chimaera. Some forms of variegation are due to a virus infection as in *Abutilon striatum Thompsonii* (5). Some types of virus infection cause a marked chlorosis of the leaf; sugar beet yellows being a case of such infection. In the case of June Yellows in Auchincruive Climax strawberry, no virus has so far been identified and this form of chlorosis has been suggested to be due to "genetical breakdown" (10).

Julius Sachs, using his classic method of growing plants in water culture, first demonstrated that plants required iron for chlorophyll formation; the application of an iron salt to the chlorotic leaves restoring the green colour.

Olsen (8) observed that the leaves of chlorotic plants invariably contained more phosphorus than did normal green plants and was of the opinion that iron in chlorotic plants was immobilized as insoluble ferric phosphate.

The discovery that chlorotic leaves often contained more iron than did green leaves tended to obscure Olsen's findings. This condition was often observed in plants growing on highly calcareous soils (17), and this type of chlorosis was accordingly termed "lime-induced." Lindner & Harley (6) were able to show that in lime-induced chlorosis there existed a definite ratio between the calcium and potassium contents of the leaves, healthy green leaves having high ratios while in chlorotic leaves the ratio was invariably low. For purposes of comparison they analysed the white and green leaves of Spiraea as an example of genetical chlorosis but could find no pertinent relation in the constituents of the ash.

De Kock and Strmecki (2), studying the growth-promoting effect of a lignite, obtained results which clearly indicated the existence of a phosphorus-iron ratio effect in mustard, the ratio being higher for chlorotic plants, while very low values indicated chlorosis due to iron toxicity. It has similarly been found that in mustard plants grown at pH 7.8 in the presence of various forms of iron, the trend in phosphorus-iron ratio was maintained, chlorotic plants having consistently higher ratios (1).

From examination of the work of other authors in which the iron and phosphorus contents of green and chlorotic leaves were given (6, 13, 14, 16, 17), it appeared that the same trend in the phosphorus-iron ratio occurred irrespective of the cause of chlorosis. Similarly, symptoms of phosphorus deficiency (3), or phosphorus toxicity (12) were in accordance with the expected phosphorus-iron ratio in each case. An examination of chlorosis due to genetical and virological causes was therefore undertaken to ascertain the trend in phosphorus-iron and calcium-potassium ratios.

Yellow, variegated and green leaves were taken from variegated herbs and shrubs in the gardens of the Macaulay Institute and from various greenhouses. These were washed in distilled water and gently dried with a clean glass cloth. If the leaves were sufficiently culticularised, as in Ilex, they were rubbed clean. They were dried at 80° C for 48 hours and then ashed in a muffle furnace at 460° C overnight. The ash was fused in "Analar" sodium carbonate in a platinum crucible and dissolved in redistilled hydrochloric acid. Iron was determined colorimetrically by $\alpha\alpha'$ dipyridyl and phosphorus by developing the phosphomolybdate blue complex with hydrazine. Calcium and potassium were determined by flame photometer (7).

The leaves analysed were taken from *Abutilon striatum Thompsonii; Bougainvillea glabra variegata; Pelargonium Zonale* var. Flower of Spring; *P. zonale* var. Rob Roy; *Ilex aquifolium* (4 variegated varieties) *Spiraea japonica* var. Anthony Waterer; *Sambucus nigra variegata.*

Table I gives the results of analyses for phosphorus, iron, calcium and potassium contents of the above leaves, together with the calculated phosphorus-iron and calcium-potassium ratios. It is clear that the value of phosphorus-iron ratio follows the degree of chlorosis observed, yellow leaves having the highest, variegated leaves intermediate and green leaves the lowest values. The calculated calcium-potassium ratios also show a definite correlation with the degree of chlorosis, being low for chlorotic leaves and high for green leaves.

The phosphorus-iron ratios for yellow and green leaves of Spiraea given by Lindner and Harley (6) do not substantiate those quoted here, the iron values for the chlorotic leaves being high. The calcium-potassium ratios do, however, agree with those here, as the authors themselves point out. Values for the iron content of yellow and green leaves of a variegated variety of pineapple (*Ananas comosus*) given by Sideris, Young and Krauss (13) are in line with the figures in table I.

Healthy and chlorotic leaves of the strawberry var. Auchincruive Climax affected with June Yel-

[1] Received November 30, 1954.

TABLE I

ANALYSIS OF THE ASH OF LEAVES OF VARIEGATED PLANTS

PLANTS	% P			% Fe			P/Fe		
	G*	V*	Y*	G	V	Y	G	V	Y
Bougainvillea glabra	...	1.02	2.35	...	0.072	0.103	...	14.15	22.9
Sambucus nigra	3.17	3.75	6.23	0.22	0.171	0.227	14.45	21.9	27.4
Ilex aquifolium, Spiny Silver	2.84	3.97	...	0.092	0.054	....	30.9	73.5	...
" " Dentate Yellow	2.81	3.97	4.05	0.054	0.057	0.050	43.9	66.5	81.0
" " Spiny Yellow	2.68	3.36	...	0.078	0.042	....	34.4	80.0	...
" " Round Yellow	2.37	3.74	...	0.084	0.050	....	28.2	74.8	...
Pelargonium zonale, Granite City	3.16	4.83	6.06	0.195	0.225	0.240	16.2	21.5	25.3
" " Flower of Spring	4.38	4.46	...	0.200	0.097	....	21.9	45.1	...
Spiraea japonica, Anthony Waterer	2.1	4.3	5.8	0.102	0.107	0.129	20.6	40.2	45.0
Abutilon striatum Thompsoni	1.21	2.23	2.57	0.098	0.074	0.082	12.3	30.0	31.4

PLANTS	% Ca			% K			Ca/K		
	G	V	Y	G	V	Y	G	V	Y
Bougainvillea glabra	...	48.2	9.6	...	14.2	26.8	...	3.4	0.36
Sambucus nigra	7.4	3.9	1.3	25.7	25.8	30.0	0.29	0.15	0.04
Ilex aquifolium, Spiny Silver	14.6	3.8	..	14.9	29.7	...	0.98	0.13	...
" " Dentate Yellow	16.4	11.8	7.3	12.9	20.7	25.6	1.27	0.57	0.29
" " Spiny Yellow	14.3	6.0	..	17.9	27.2	...	0.80	0.22	...
" " Round Yellow	23.6	5.4	..	14.1	20.5	...	1.7	0.26	...
Pelargonium zonale, Granite City	16.4	7.2	5.1	22.0	25.8	29.1	0.75	0.28	0.18
" " Flower of Spring	17.2	13.6	..	15.5	18.3	...	1.1	0.74	...
Spiraea japonica, Anthony Waterer	23.9	...	4.3	15.1	...	28.2	1.6	...	0.15
Abutilon striatum Thompsoni	35.5	...	..	4.4	...	...	8.1	...	...

* G = green; V = variegated; Y = yellow.

lows were analysed and gave phosphorus-iron ratios of 7.9 for normal leaves and 12.1 for yellowed leaves. This once more shows that the phosphorus-iron ratio follows the symptoms observed. Chlorosis due to toxicity of heavy metals will be discussed elsewhere but as indicated above (e.g., Reuther, Smith and Specht, 11) the phosphorus-iron ratio is in accordance with the symptoms of chlorosis.

Phosphorus and iron analyses of the needles of fir, *Abies alba* attacked by the aphid, *Adelges nusslini* C.B. which causes severe chlorosis and death of the affected trees (15) are presented in table II.

From the foregoing tables it would appear that chlorotic and green plants can be distinguished from each other by their phosphorus-iron or calcium-potassium ratios. It is probable that other ratios such as magnesium-potassium can similarly be calculated and shown to be significant.

Pierce & Appleman (9) have demonstrated that a balance exists between cations and organic acids in plant leaves. Organic acids are generated in the respiratory cycle of the cell, the rate of which is controlled by the iron enzymes (4) which all possess cytochrome structures. De Kock & Farmer (unpublished results) have shown that total cytochromes and chlorophyll of leaves are also correlated, reduction in chlorophyll always being accompanied by reduction in total cytochrome no matter what the cause of the chlorosis. It seems possible that the two above ratios are maintained in the plant cell by the balance of organic acids, which are in turn largely dependent on the amount of iron available for synthesis of respiratory pigments. Evidence in support of this view is provided by the work of Lindner & Harley (6) who analysed chlorotic leaves of Bartlett pear and leaves on the same tree which had been greened by soil treatment with iron sulphate. The phosphorus-iron ratio in the chlorotic leaves was 53.7 while that of the greened leaves was 9.14. The decrease in the ratio had been largely brought about by a decrease in the amount of phosphorus in the leaf, showing that as the iron content of the leaf increased, phosphorus apparently migrated out of the leaf cells. It will be noticed moreover that the calcium-potassium ratio which is low in the chlorotic leaf changes to a high value in the greened leaf. There is little doubt then that the plant cell exercises some control over its mineral constituents.

TABLE II

ANALYSIS OF FIR NEEDLES ATTACKED BY APHIDS

DRY MATTER	% P	% Fe	P/Fe
Healthy	0.31	0.035	8.9
Chlorotic	0.25	0.016	15.6

SUMMARY

The ratio of phosphorus to iron and of calcium to potassium of chlorotic leaves of variegated plants show the same trends from those of normal leaves as is shown by other forms of chlorosis, the phosphorus-

iron ratio being higher, and the calcium-potassium ratio being lower than for normal green leaves. This also applies to chlorosis due to virological or pathological causes.

The writers wish to express their appreciation to Dr. R. L. Mitchell for the flame photometric results of calcium and potassium and assistance with the manuscript.

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News and Notes

George James Peirce

George James Peirce died on October 15, 1954, aged 86. With him, American plant physiology lost one of its last remaining links with the great German tradition which so influenced its development in the first part of the twentieth century.

Peirce was born of American parents in Manila in 1867, attended the Lawrence Scientific School at Harvard from which he graduated in 1890. He was then awarded one of the first American travelling stipends, the Parker Fellowship of Harvard. His Wanderjahre included a year each in Bonn and Munich, where he studied, like so many other American botanists, with Strassburger and Goebel. Then he moved to the great laboratory where Pfeffer was shaping the form and future of the new science of Plant Physiology. Peirce won his Ph.D. *summa cum laude* at Leipzig in 1894, his thesis being on the physiology of the parasitic flowering plant, *Cuscuta*, the dodder.

Returning to America, Peirce again spent a year at Cambridge, applied for the position at Smith College which was won by Ganong, and in 1895 went to Indiana University, in the then small and rather southern town of Bloomington. Indiana had just before this supplied David Starr Jordan, Douglas Houghton Campbell, and several other faculty members to the new university which Leland Stanford had endowed on his Palo Alto Ranch in California. It was not surprising, therefore, that, as botany grew at Stanford, Peirce should join Campbell. He became instructor in 1897, assistant professor in 1900, and remained in the department for all his career, taking over much administrative responsibility long before he became chairman in 1925. He retired in 1933.

The key to Peirce's research and teaching was in his title: Professor of Botany and Plant Physiology, which he always preferred to retain. While leaving to colleagues the exactitudes of taxonomy, he loved plants as such, especially out-of-doors, and he was more concerned with their relations to environment, their means of survival and response, and the correlation of structure and function, then he was with biochemistry, or the physical chemistry of protoplasm. It was the categories of Pfeffer's physiology which he taught, and incorporated in his own "Textbook of

Plant Physiology," which was published in 1903 and revised in 1926. His last book was a very personalized little Laboratory Manual (1932), which raised more questions than it answered, and taught an approach rather than methods. Indeed, as a teacher, he imparted an attitude toward the life of plants (and of men), which students remembered long after the facts of science had changed, or altered in interest.

His teaching of physiological plant anatomy was inspired by his teacher Goodale at Harvard; students in this course recall Peirce's skill at free-hand sections, and the amount he could show about freshly cut xylem and phloem with iodine and simple stains. He took his classes out-of-doors as much as possible, making the most of California's genial climate: asking how tar-weed and eucalyptus got their water in the desiccated autumn; watching the germination of buckeye seeds, and the growth of grass and miner's lettuce in the wet winter; marvelling at the incredible rush of blossom and seeding in the warm spring. Summer usually took him to his beloved Fallen Leaf Lake in the high Sierra, where he built cottages and pondered the struggles of mountain juniper to grow on barren granite.

He did not deal exclusively with higher plants; he taught courses on marine algae at Pacific Grove, and always remained interested in the physiology of the remarkable brine alga, *Dunaliella*, which lives in the salt ponds around San Francisco Bay. He studied the semi-independent sporophyte of Anthoceros and the morphogenetic effects of light and gravity on liverworts. But his most abiding interest was doubtless in the problem of "how water goes up a tree." He thought and experimented much on this, and devised ingenious, simple tests of some hypotheses bearing on this question—which still, perhaps, needs definite answer. It was a special pleasure to him then, when H. H. Dixon visited California, and again when he, with Mrs. Peirce, could return the visit in Dublin after his retirement.

Both his love of plants and his strong social sense doubtless contributed to another interest, that of smoke and dust damage to native and cultivated plants. For many years he studied the effects of smelter fumes near San Francisco, and in Montana and Utah; and the deposition of dust on leaves near cement plants. His last notebook contains observations on the latter, made only a year before his death. He was involved as a witness in several suits on such matters—which will doubtless continue to occupy plant physiologists in California.

Some of Peirce's better known students include O. L. Sponsler, Flora Murray Scott, L. G. M. Baas-Becking, Harry A. Borthwick, Delzie Demaree. He was a Fellow of the American Academy of Arts and Sciences, and one of the early members of the American Society of Plant Physiologists. Befitting his interests in water relations, he served on the committee which established the terms of the Stephen Hales Award. He was given the Charles Reid Barnes Life Membership in 1936. In 1932 he was president of the Botanical Society of America.

Failing sight dimmed his last year, but never his twinkling eye or ready, teasing wit. It was an honor to succeed him and to share with his family his "honor, generosity, and high-minded mirth." A Berkeley associate addressed him on his eighty-fifth birthday as "a very rich man: rich in the affection of students and the respect of hosts of friends and colleagues." The President of Stanford said: "The gentleness that was in him affected all with whom he spoke, and characterized everything that he did. His presence brought sunshine; memory of him cannot fail to keep it bright."—L. R. Blinks.